(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
9 August 2001 (09.08.2001)

PCT

(10) International Publication Number
WO 01/57245 A2

(51) International Patent Classification[7]: C12Q 1/68

(21) International Application Number: PCT/BE01/00017

(22) International Filing Date: 5 February 2001 (05.02.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

0002533.8	4 February 2000 (04.02.2000)	GB
0101011.5	15 January 2001 (15.01.2001)	GB

(71) Applicant *(for all designated States except US)*: **K.U.LEUVEN RESEARCH & DEVELOPMENT** [BE/BE]; Groot Begijnhof, Benedenstraat 59, B-3000 Leuven (BE).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **WITVROUW, Myriam** [BE/BE]; Wandeleerstraat 27, B-3201 Langdorp (BE). **FIKKERT, Valery** [BE/BE]; Nindsebaan 43, B-3140 Keerbergen (BE). **PANNECOUQUE, Christophe** [BE/BE]; Sint-Jansbergsesteenweg 70, B-3001 Leuven (Heverlee) (BE). **CHEREPANOV, Peter** [RU/BE]; Brusselsestraat 128, B-3000 Leuven (BE). **VAN LAETHEM, Kristel** [BE/BE]; Hoogstraat 176, B-2830 Tisselt (BE). **DE CLERCQ, Erik** [BE/BE]; Parklaan 9, B-3360 Lovenjoel (BE). **VANDAMME, Anne-Mieke** [BE/BE]; Beatrijslaan 9, B-3110 Rotselaar (BE). **DEBYSER, Zeger** [BE/BE]; Korbeek-Losestraat 108, B-3001 Heverlee (BE).

(74) Agent: **ROELANTS, Ivo**; K.U.Leuven Research and Development, Groot Begijnhof, Benedenstraat 59, B-3000 Leuven (BE).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/57245 A2

(54) Title: HIV-1 RESISTANCE ASSAY

(57) Abstract: Present invention involves a recombination assay for the HIV envelope genes, *gp120*, *gp41*, and *gp160*. It further involves *env*-deleted proviral clones, the optimization of the PCR amplification of the corresponding *env*-genes and the subsequent sequencing of these genes. These techniques have been applied on several HIV-1(NL4.3) strains selected *in vitro* in the presence of increasing concentrations of inhibitors of HIV entry and evaluated for the phenotypic resistance of these recombined viruses. This phenotypic resistance has been correlated with genotypic resistance. Present invention also involves a recombination assay for the integrase gene.

HIV-1 RESISTANCE ASSAY

The current treatment of human immunodeficiency virus (HIV) infection focuses primarily on inhibition of the viral enzymes reverse transcriptase (RT) and protease (PRO). Still, combination therapies based on these compounds are not able to completely suppress virus replication. Residual replication in the presence of the selective pressure of antiviral drugs allows the emergence of drug-resistant strains, resulting in therapeutic failure (Larder *et al.*, 1998, Vandamme *et al.*, 1999a). To assess the implications of HIV drug resistance on patient management, assays to evaluate drug-resistance of clinical HIV isolates against the current RT and PRO inhibitors are becoming more widely used (Kuritzkes, 1999). Whereas genotypic drug resistance assays are fast and relatively cheap to monitor the presence of known resistance-related mutations, only phenotypic assays can measure the overall viral response to the drug and can determine the possible synergistic or antagonistic effects of a combined set of mutations. The fastest and most reproducible replication-based phenotypic assay is the 'Recombinant Virus Assay', devised by Kellam and Larder (Kellam and Larder, 1994). The Recombinant Virus Assay (RVA) is based on the generation of viable virus by homologous recombination of a polymerase chain reaction (PCR)-pool of patient-derived viral RT gene into a RT-deleted proviral clone of a cell-culture adapted HIV-1 strain. This technique was designed for the susceptibility testing of clinical isolates towards RT inhibitors. Maschera *et al.* (1995) extended this method to the protease gene. A combined RT and PRO RVA has also been developed (Hertogs *et al.*, 1998). The resulting recombined virus retains the sensitivity towards RT and/or protease inhibitors of the clinical isolate, while the inhibition of the HIV-induced cytopathicity can now be measured by fast and cheap tests, designed for laboratory strains in a semi-automated manner.

Since virus drug resistance is the main cause of therapeutic failure, the development of new drugs, preferentially acting on new targets in the HIV replication cycle is of high priority in anti-HIV research. A potentially powerful target, in addition to RT and PRO, is HIV entry, the first event in the virus replicative cycle. Several compounds that inhibit viral entry have been described. These molecules act at different stages of HIV entry. Polyanionic structures in general inhibit the binding of gp120 to CD4 on the surface of the host cell (Baba *et al.*, 1988). HIV co-receptor antagonists have also been described as HIV

inhibitors. The low molecular mass bicyclams are highly potent and selective CXCR4 antagonists (De Clercq *et al*, 1992, 1994; De Vreese *et al.*, 1996a, b; Schols *et al.*, 1997). The bicyclam AMD3100 is currently subject of phase II clinical trials (Hendrix *et al.*, 1999, Vartanian, 2000). AMD3100 not only inhibits the replication of X4 strains, but may also prevent the switch from the less pathogenic R5 strains to the more pathogenic X4 HIV strains (Esté *et al.*, 1999). Other inhibitors of viral entry interact with the fusion process itself. T-20, a synthetic peptide segment of the ectodomain of gp41, has now proceeded to phase II clinical trials (Kilby *et al.*, 1998, Drucker, 2000).

Another interesting target in the replicative cycle of HIV is the viral enzyme integrase, since integration is an indispensable step during HIV replication and no human counterpart of the enzyme is known. Several anti-HIV compounds were initially proffered as integrase inhibitors. However, only one authentic series of integrase inhibitors, the diketo acids (e.g. L-731,988) have been identified until now (Hazuda *et al.*, 2000).

Given that the entry inhibitors AMD3100 and T-20 are already introduced in clinical studies, methods to evaluate the susceptibility of clinical virus isolates towards inhibitors of HIV entry are required. Therefore, we have developed recombination assays for the HIV envelope genes, *gp120*, *gp41* and *gp160*. Here, we describe the construction of the different *env*-deleted proviral clones, the optimization of the PCR amplification of the corresponding *env*-genes and the subsequent sequencing of these genes. Furthermore, we applied these techniques on several HIV-1(NL4.3) strains selected *in vitro* in the presence of increasing concentrations of inhibitors of HIV entry and evaluated the phenotypic resistance of these recombined viruses, and correlated this phenotypic resistance with genotypic resistance.

Since HIV-1 integration is likely to be a future target for novel anti-HIV agents, we have designed as well a recombination assay for the integrase gene. We applied the technique using a wild-type HIV-1(III_B) strain to obtain a proof-of-principle regarding the recombination of the integrase gene.

ILLUSTRATIVE DESCRIPTION OF THE EMBODIMENTS

Selection of HIV-1(NL4.3) strains resistant to virus cell entry inhibitors

The HIV-1(NL4.3) strains resistant to L-CA (L-CA^{res}), BRI2923 (BRI2923^{res}) and AMD3100 (AMD3100^{res}) were selected after serial passage (23 to 63 passages) of HIV-1(NL4.3) in the presence of increasing concentrations of the respective compounds (Table 1). The experiment was initiated at a low multiplicity of infection (moi: 0.01) and a drug concentration equal to 5-fold its IC_{50}. Every 3 to 4 days the culture was monitored for the appearance of CPE. When CPE was observed, the cell-free culture supernatant was used to reinfect fresh, uninfected cells in the presence of equal or higher concentrations of the compound. When no virus breakthrough was observed, the cell culture was subcultivated in the presence of the same concentration of the compound. Gradually, after subsequent passaging, the concentration of the compound required for the prevention of virus break-trough increased significantly to 30 µg/ml for L-CA, 20 µg/ml for BRI2923 and 500 µg/ml for AMD3100 (Table 1). These final concentrations were 4- and 67 fold higher than the concentration required to inhibit the replication of wild-type HIV-1(NL4.3) by 50% (IC_{50}) for L-CA and BRI2923, respectively. For AMD3100, the final concentration was 125,000 times higher than the IC_{50} value for wild-type HIV-1(NL4.3) (Table 1).

Genotypic analysis of the gp120 genes of HIV-1(NL4.3) strains resistant to virus cell entry inhibitors

The DNA sequencing analysis of the gp120 encoding region of the drug-resistant HIV-1(NL4.3) strains revealed several mutations that were not present in the wild-type strain (Table 5). Noteworthy are the amino acid changes from serine (S) (at position 160) and threonine (T) (at position 372 and 378) in the L-CA-resistant strain towards asparagine (N). The amino acid residues S and T are possible *O*-glycosylation sites, while N is a potential site for *N*-glycosylation. These carbohydrate moieties are important for the three-dimensional structure of gp120 and are known to play an important role in the immune escape of HIV (Cheng-Mayer *et al.*, 1999).

It has been reported before that polyanionic compounds, like dextran sulfate, can select for the mutation from N to aspartic acid (D) (negatively charged) at position 295 of *gp120* (N295D) (Esté *et al.*, 1997). This mutation is also found in the L-CA^{res} strain. In contrast,

the polycationic molecule AMD3100 selects for histidine (H) (positively charged) at this position. The five amino acid deletion (FNSTW) in the V4 loop is associated with *in vitro* resistance towards various binding and fusion inhibitors: it is found in the BRI2923- and AMD3100-resistant strains. AMD3100 resistance selection elicits extensive mutations in the V3 loop, known to be important for interaction with the CXCR4 co-receptor (Verrier *et al.*, 1999), a logical strategy for the virus to escape inhibitors of the virus-CXCR4 interaction.

Genotypic analysis of the gp41 genes of HIV-1(NL4.3) strains resistant to virus cell entry inhibitors

The genotypic analysis of *gp41* revealed mutations only for the BRI2923^{res} strain, and not for the L-CA- and AMD3100^{res} strains (Table 6). The substitutions of alanine (A) by valine (V) at position 22 and of leucine (L) by serine (S) at position 33 of the *gp41*-gene were found in the BRI2923^{res} strain, as well as the mixtures proline/leucine (P/L) and alanine/threonine (A/T) at the positions 216 and 308 of the *gp41* gene, respectively.

Evaluation of phenotypic resistance and cross-resistance of the selected HIV-1 strains resistant to virus cell entry inhibitors

We evaluated the antiviral activity of the virus cell entry inhibitors L-CA, BRI2923, AMD3100 and T-20 against resistant HIV-1(NL4.3)-derived strains that were selected *in vitro* in the presence of L-CA, BRI2923 and AMD3100 (L-CA^{res}, BRI2923^{res} and AMD3100^{res} strains) (Table 7). Resistance of the selected strain against the drug used for selection (shown in bold on the diagonal line in Table 7) was very high for the AMD3100^{res} strain, but much lower for BRI2923^{res} and L-CA^{res} strains. This can be explained by lower final concentrations of the latter products during resistance selection (Table 1).

The L-CA^{res} strain showed little cross-resistance towards the polyanionic compound BRI2923, while the BRI2923^{res} strain was highly cross-resistant to the fusion inhibitor T-20, but still sensitive to L-CA and AMD3100. The AMD3100^{res} strain displayed cross-resistance towards BRI2923, but not towards L-CA and T-20.

L-CA only lost inhibitory activity against the L-CA^{res} strain. BRI2923 lost activity towards all resistant strains evaluated, although this was less pronounced in case of the L-CA^{res}

strain. The CXCR4 antagonist AMD3100 only lost antiviral activity (158-fold) against the AMD3100^{res} strain, whereas T-20 was less active against the BRI2923^{res} strain only.

Evaluation of resistance and cross-resistance of the different env-recombined viruses

Evaluation of resistance and cross-resistance of the gp120-recombined viruses

The sensitivity of the L-CA^{res} *gp120*-recombined strain, as well as the AMD3100^{res} *gp120*-recombined strain matched the sensitivity of the *in vitro* selected L-CA^{res} and AMD3100^{res} strains for all compounds evaluated (L-CA, BRI2923, AMD3100 and T-20) (Table 7 and 8). However, *gp120*-recombination of the BRI2923^{res} strain in the wild-type background of NL4.3 did not reproduce the resistance phenotype of the *in vitro* selected strain against BRI2923 and T-20. The BRI2923^{res} *gp120*-recombined strain displayed the same sensitivity as the NL4.3 wild-type *gp120*-recombined strain to these virus cell entry inhibitors (Table 8). The wild-type *gp120*-recombined strain, as well as the L-CA^{res} *gp120*-recombined and AMD3100^{res} *gp120*-recombined viruses, showed a 5- to 10-fold lower sensitivity to the inhibitory effect of T-20 in comparison to the NL4.3 wild-type, the L-CA^{res} and the AMD3100^{res} *in vitro* selected strains.

Evaluation of resistance and cross-resistance of the gp41-recombined viruses

The L-CA^{res}-, BRI2923^{res}- and AMD3100^{res} *gp41*-recombined viruses displayed the same sensitivity to L-CA, BRI2923 and AMD3100 as the wild-type HIV-1(NL4.3) *gp41*-recombined strain. However, T-20 lost its antiviral activity against the BRI2923^{res} *gp41*-recombined strain, but not against the other *gp41*-recombined strains; i.e. NL4.3^{WT}, L-CA^{res} and AMD3100^{res} (Table 9).

Evaluation of resistance and cross-resistance of the gp160-recombined viruses

The recombination of the *gp160*-gene of the AMD3100^{res} strain fully rescued the resistance phenotype of the original *in vitro* selected strain. This finding is totally in line with the expectations, since *gp120*-recombination was already sufficient to rescue the resistance profile of this *in vitro* selected virus towards the four compounds evaluated. The BRI2923^{res} *gp160*-recombined strain, however, displayed wild-type sensitivity to BRI2923, but not to T-20. L-CA and AMD3100 did not lose activity against the *in vitro* selected BRI2923^{res} strain, nor against the BRI2923^{res} *gp160*-recombined strain. *gp160*-recombination has not been carried out for the L-CA^{res} strain (Table 10).

Genotypic analysis of the gag genes of HIV-1(NL4.3) strains resistant to virus cell entry inhibitors

The genotypic analysis of *gag* revealed mutations only for the BRI2923^{res} strain, and not for the L-CA^{res} and AMD3100^{res} strains (Table 11). The substitutions of glutamic acid (E) by lysine (K) at position 12 and of lysine (K) by arginine (R) at position 27, as well as the mutations from valine (V) to isoleucine (I) at position 35 and from glycine (G) to arginine (R) at position 62 were found in the BRI2923 ^{res} strain. In addition, the mixture of alanine/glutamic acid (A/E) was found at position 125 of the *gag* gene in the BRI2923^{res} strain. The latter mutation is located in the p24 coding sequences of the *gag* gene.

Recombination of the integrase gene

To obtain a proof-of principle that this chimeric virus technology could also be applied to the integrase gene, the integrase gene of the HIV-1(III_B) strain was placed in the genetic background of the HIV-1(NL4.3) integrase-deleted clone. The homologous recombination resulted in viable virus and recombination was demonstrated by sequencing analysis. The sensitivity of the resulting virus was evaluated for a nucleoside RT inhibitor (zidovudine), a non-nucleoside RT inhibitor (nevirapine), a protease inhibitor (ritonavir) and an integrase inhibitor [L-731,988 (diketo acid)]. As expected, the antiviral activities of these compounds against the HIV-1(III_B)-integrase-recombined NL4.3 strain, the HIV-1(III_B) and the HIV-1(NL4.3) strains were identical (Table 12).

Examples

Example 1. *Compounds, Cells and Virus strains*

L-Chicoric Acid (L-CA) was synthesized according to the method described elsewhere (Zhao and Burke, 1998). The origin of the dendrimer BRI2923 is described elsewhere (Witvrouw *et al.*, 2000). The bicyclam AMD3100 was kindly provided by Geoffrey Henson, AnorMED (Langley, BC, Canada) and was synthesized as described (Bridger *et al.*, 1995). T-20 was a gift from S. Brown (AIDS Research Alliance, West Hollywood, CA).

MT-4 cells (Miyoshi *et al.*, 1982) were grown and maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2mM L-glutamine, 0.1% sodium bicarbonate and 20 µg gentamycin per ml.

The HIV-1 NL4.3 strain is a molecular clone (Adachi *et al.*, 1986) obtained from the National Institutes of Health (Bethesda, MD). The HIV-1(NL4.3) strains resistant to the entry inhibitors L-CA (L-CA^{res}), BRI2923 (BRI2923^{res}) and AMD3100 (AMD3100^{res}) were selected after serial passage of HIV-1(NL4.3) in the presence of increasing concentrations of the respective compounds (Table 1). The AMD3100^{res} strain was previously selected in our laboratory (De Vreese *et al.*, 1996a,b). The origin of HIV-1(III_B) has been described (Popovic *et al.*, 1984).

Example 2: Drug susceptibility assay

The inhibitory effect of the various antiviral drugs on the HIV-induced cytopathicity of the different strains in human lymphocyte MT-4 cell culture was determined by the MTT assay (Pauwels *et al.*, 1988). This assay is based on the reduction of the yellow colored 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by mitochondrial dehydrogenase of metabolically active cells to a blue formazan derivative, which can be measured spectrophotometrically. The 50% cell culture infective dose ($CCID_{50}$) of the different HIV virus strains was determined by titration of the virus stock using MT-4 cells. For the drug-susceptibility assays MT-4 cells were infected with 100-300 $CCID_{50}$ of the HIV strains in the presence of five-fold dilutions of the antiviral drugs. The concentration of the compound achieving 50% protection against the cytopathic effect (CPE) of the various HIV strains, which is defined as the 50% inhibitory concentration (IC_{50}), was determined.

Example 3.: Optimizing the PCR amplification and the sequencing of the different genes

PCR Amplification of gp120 encoding sequences

MT-4 cells were infected with HIV laboratory strains resistant to virus cell entry inhibitors. DNA extraction of proviral DNA was performed using the QIAamp blood Kit (Qiagen, Westburg, Leusden, The Netherlands). A 2105-nucleotide base pair fragment (codons 1 to 445) of *gp120* was amplified in a nested PCR using Expand ™ High Fidelity PCR system

(Boehringer Mannheim, Roche, Germany), which is composed of an enzyme mix containing thermostable Taq DNA and Pwo DNA polymerase with 3'-5' exonuclease proofreading capacity. The outer PCR reaction was performed on a Perkin Elmer Gene Amp PCR system 9600 and the inner PCR on a Biometra Trioblock (Westburg) using the primers AV310 (5'-AGC AGG ACA TAA T/CAA GGT AGG-3' corresponding to position 5447-5467 of NL4.3) and AV311 (5' GGA GAA GTG AAT TAT ATA AG/AT ATA AAG TAG-3' corresponding to position 7630-7659 of NL4.3), followed by the primers AV312 (5'-AGA A/GGA C/TAG ATG GAA CAA GCC CCA G-3' corresponding to position 5549-5573 of NL4.3) and AV313 (5'-GAC CTG GAG GAG GAA/G ATA TGA G/AGG A-3' corresponding to position 7605-7629 of NL4.3). The outer cycling conditions were as follows: a first denaturation step of 2 min at 95°C followed by 40 cycles of 30 sec 95°C, 30 sec 50°C, 2 min 68°C. A final extension was performed at 72°C for 10 min. For the inner cycling, the following conditions were used: after 2 min at 95°C, 30 cycles of 30 sec 95°C, 30 sec 58°C, 2 min 68°C and 10 min 72°C extension (Table 2).

Sequencing of the gp120-coding region

PCR products were purified using the PCR purification kit (Qiagen, Westburg, Leusden, The Netherlands). To carry out the sequencing reaction, the ABI PRISM™ Dye terminator cycle sequencing core kit (Perkin Elmer, Brussels, Belgium) was used. The primers used to sequence the *gp120* gene were: AV304 (5'-ACA TGT GGA AAA ATG ACA TGG T-3' corresponding to position 6504-6525 of NL4.3), AV305 (5'-GAG TGG GGT TAA TTT TAC ACA TGG-3' corresponding to position 6552-6575 of NL4.3), AV306 (5'-TGT CAG CAC AGT ACA ATG TAC ACA-3' corresponding to position 6946-6969 of NL4.3), AV307 (5'-TCT TCT TCT GCT AGA CTG CCA T-3' corresponding to position 6987-7008 of NL4.3), AV308 (5'-TCC TCA GGA GGG GAC CCA GAA ATT-3' corresponding to position 7313-7336 of NL4.3), AV309 (5'-CAG TAG AAA AAT TCC CCT CCA CA-3' corresponding to position 7333-7355 of NL4.3) and AV313 (5'-GAC CTG GAG GAG GAA/G ATA TGA G/AGG A-3' corresponding to position 7605-7629 of NL4.3) (Table 2). The samples were loaded on the ABI PRISM 310 Genetic Analyser (Perkin Elmer, Brussels, Belgium). The sequences were analyzed using the software program Geneworks 2.5.1 (Intelligenetics Inc., Oxford, UK).

PCR Amplification of gp41 encoding sequences

A 1560-nucleotide base pair fragment (corresponding to position 7353-8913 of NL4.3) was amplified by PCR using Expand ™ High Fidelity PCR system. The PCR reaction was performed on a Biometra Trioblock using the primers AV320 (5'-ATT GTA/G GAG GA/GG AAT TTT TCT ACT G-3' corresponding to position 7353-7378 of NL4.3) and AV321 (5' TTG CTA/G CTT GTG ATT GCT/C CCA TG-3' corresponding to position 8890-8913 of NL4.3). The cycling conditions were as follows: a first denaturation step of 2 min at 95°C followed by 40 cycles consisting of 15 sec 95°C, 30 sec 55°C, 2 min 68°C. A final extension was performed at 72°C for 10 min (Table 2).

Sequencing of the gp41-coding region

The primers used to sequence the *gp41* gene were: AV322 (5'-AAG CAA TGT ATG CCC CTC C-3' corresponding to position 7518-7537 of NL4.3), AV323 (5'-CTG CTC CC/TA AGA ACC CAA-3' corresponding to position 7764-7782 of NL4.3), AV324 (5'-GGC AAA GAG AAG AGT GGT-3' corresponding to position 7723-7741 of NL4.3), AV325 (5'-GTA TCT TTC CAC AGC TAG-3' corresponding to position 7946-7964 of NL4.3), AV326 (5'-TTG GGG T/CTG CTC TGG AAA AC-3' corresponding to position 8008-8028 of NL4.3), AV327 (5'-TTT TAT ATA CCA CAG CCA-3' corresponding to position 8237-8255 of NL4.3), AV328 (5'-ATA ATG ATA GTA GGA GG-3' corresponding to position 8279-8296 of NL4.3), AV329 (5'-GTC CCA GAA GTT CCA CA-3' corresponding to position 8549-8566 of NL4.3), AV330 (5'-GGA G/ACC TGT GCC TCT TCA-3' corresponding to position 8505-8523 of NL4.3), AV331 (5'-TCT CAT TCT TTC CCT TA-3' corresponding to position 8825-8842 of NL4.3) (Table 2).

PCR Amplification of the gp160 encoding sequences

A 3577 nucleotide base pair fragment (corresponding to position 5447-9024 of NL4.3) was amplified by PCR using Expand ™ High Fidelity PCR system. The PCR reaction was performed on a Biometra Trioblock using the primers AV310 (5'-AGC AGG ACA TAA T/CAA GGT AGG-3' corresponding to position 5447-5467 of NL4.3) and AV319 (5' -GCT G/C CC TTA/G TAA GTC ATT GGT CT-3' corresponding to position 9001-9024 of NL4.3). The cycling conditions were as follows: a first denaturation step of 2 min at 95°C followed by 40 cycles consisting of 15 sec 95°C, 30 sec 55°C, 3 min 40 sec 68°C. A final extension was performed at 72°C for 10 min (Table 2).

PCR Amplification of gag encoding sequences

A 1424-nucleotide base pair fragment (corresponding to position 59-1483 of NL4.3) was amplified by PCR using AmpliTaq (Perkin Elmer, Brussels, Belgium). The PCR reaction was performed on a Biometra Trioblock using the primers AV14 (5'-CTC TCT CGA CGC AGG ACT CGG CTT GCT GAA-3') corresponding to position 59-88 of NL4.3 and BVR2 (5'-GCC AG/AA TC/TT TCC CTA AAA AAT TAG CC-3') corresponding to position 1457-1483 of NL4.3. The cycling conditions were as follows: a first denaturation step of 2 min at 95°C followed by 40 cycles consisting of 15 sec 95°C, 30 sec 55°C, 90 sec 72°C. A final extension was performed at 72°C for 10 min (Table 3).

Sequencing of the gag-coding region

The primers used to sequence the p17 and p24 encoding regions of the *gag* gene were: AV13 (5'-CTG CGA ATC GTT CTA GCT CCC TGC TTG CCC -3') corresponding to position 245-274 of NL4.3, AV26 (5'-GCT ATG TCA CTT CCC CTT GGT TCT C -3') corresponding to position 829-853 of NL4.3, AV54 (5'-GAG ACC ATC AAT GAG GAA GCT GC-3') corresponding to position 774-796 of NL4.3, AV58, (5'-GGC TAA TTT TTT AGG-3') corresponding to position 1457-1471 of NL4.3, AV159 (5'-GGG GTT AAA TAA AAT AGT AAG-3') corresponding to position 971-991 of NL4.3, AV103 (5'-GCC ATA TCA CCT AGA ACT TT-3') corresponding to position 603-622 of NL4.3, GAG-1 (5'-AGA AAC ATC AGA AGG CTG TAG-3') corresponding to position 320-340 of NL4.3, BVR3 (5'-TTT CCA ACA GCC CTT TTT CCT AG-3') corresponding to position 1368-1390 of NL4.3, BVR4 (5'-AGA CAC CAA G/AGA AGC-3') corresponding to position 352-366 of NL4.3, BVR5 (5'-TCC T/CTC TGA TAA TGC TG-3') corresponding to position 669-685 of NL4.3 and BVR6 (5'-TAG AAG AAA TGA TGA CAG C-3') corresponding to position 1195-1213 of NL4.3 (Table 3)

PCR Amplification of integrase encoding sequences

A 2578-nucleotide base pair fragment (corresponding to position 3003-5581 of NL4.3) was amplified by PCR using Expand ™ High Fidelity PCR system. The PCR reaction was performed on a Biometra Trioblock using the primers MW3 (5'-TAT GTA GGA/G TCT GAC/T TTA GAA ATA GGG-3' corresponding to position 3117-3144 of NL4.3) and MW4 (5'-TAA CAC TAG GCA A/GAG GTG GCT T-3' corresponding to position 5524-5546 of NL4.3). The cycling conditions were as follows: a first denaturation step of 2

min at 95°C followed by 40 cycles consisting of 15 sec 95°C, 30 sec 60°C, 3 min 68°C. A final extension was performed at 72°C for 10 min (Table 4).

Sequencing of the integrase-coding region

The primers used to sequence the integrase gene were: IN-PCRA (5'-GGAGGAAATGAACAAGTAGAT-3' corresponding to position 4181-4202 of NL4.3), HP4392C (5'-TCTACTTGTCCATGCATGGCTTC-3' corresponding to position 4377-4399 of NL4.3), IN-SEQ1 (5'-TTAAGATGTTCAGCCTGATCT-3' corresponding to position 4733-4753 of NL4.3), IN-SEQ2 (5'-CATTGTCTGTATGTACTGTTT-3' corresponding to position 4567-4587 of NL4.3), IN-SEQ3 (5'-GGATATATAGAAGCAGAAGTAA-3' corresponding to position 4479-4500 of NL4.3), IN-SEQ4 (5'-GAACATCTTAAGACAGCAGTA-3' corresponding to position 4743-4753 of NL4.3), IN-SEQ5 (5'-AAGCTCCTCTGGAAAGGTGAA-3' corresponding to position 4953-4973 of NL4.3), and IN-PCRB (5'-CCTTGAAATATACATATGGTG-3' corresponding to position 5125-5145 of NL4.3) (Table 4).

Example 4. : Construction of the different deleted clones.

Construction of the gp120-deleted clone.

The proviral molecular clone pNL4.3 (Adachi *et al.*, 1986) was used as starting material. This clone consists of the plasmid pUC18, wherein the complete HIV-1 genome flanked with chromosomal DNA is inserted. To generate the *gp120*-deleted clone, pNL4.3 was digested with the restriction enzymes *Sal* I and *Mam* I. The vector was purified by gel extraction (using β-agarase I) and subsequent phenol/chloroform extraction. A 23/19 base pair linker containing the *Sal* I and *Mam* I restriction sites was ligated into the vector to recirculize the plasmid (Fig. 1a). Escherichia coli bacteria (DH5α) were transformed with this *gp120*-deleted clone. For use in recombination experiments large-scale plasmid DNA preparations were linearized by *Sal* I digestion and recombined with PCR amplified *gp120* genes from the selected strains resistant to virus cell entry inhibitors (Fig. 1b).

Construction of the gp41-deleted clone.

To generate the *gp41*-deleted clone, pNL4.3 was digested with the restriction enzymes *Mam* I and *Cel* II. A 22/25 base pair linker containing the *Xba* I restriction site was ligated

into the vector to recirculize the plasmid (Fig. 2a). For use in recombination experiments large-scale plasmid DNA preparations were linearized by *Xba* I digestion and recombined with PCR amplified *gp41*-genes derived from the HIV strains selected in the presence of virus cell entry inhibitors (Fig. 2b).

Construction of the gp160-deleted clone.

pNL4.3 was digested with the restriction enzymes *Sal* I and *Cel* II to generate the *gp160*-deleted clone. A 28/28 base pair linker containing the *Sal* I and *Cel* II restriction sites was ligated into the vector to recirculize the plasmid (Fig. 3a). For use in recombination experiments large-scale plasmid DNA preparations were linearized by *Sal* I digestion and recombined with PCR amplified *gp160*-genes derived from the HIV strains selected in the presence of virus cell entry inhibitors (Fig. 3b).

Construction of the integrase-deleted clone.

To generate the *integrase*-deleted clone, pNL4.3 was digested with the restriction enzymes *Pin* AI and *Van* 91 I. A 21/14 base pair linker containing the *Xba* I restriction site was ligated into the vector to recirculize the plasmid (Fig. 4a). For use in recombination experiments large-scale plasmid DNA preparations were linearized by *Xba* I digestion and recombined with PCR amplified *integrase* genes from certain virus strains (Fig. 4b).

Construction of the gag-deleted clone.

pNL4.3 has been digested with the restriction enzymes *BssH* II and *Spe* I to generate the *p17*-deleted clone. A linker sequence, which contains besides the *BssH* II and *Spe* I restriction sites, the rest of the packaging signal and the novel *Mlu* I restriction site, has been created by means of PCR amplification (Figures 6 and 7). PCR amplification of the pNL4.3 sequence with the primers AV14 and PC-VAB, followed by digestion of the resulting PCR product with the endonucleases *BssH* II and *Spe* I resulted in a unique linker sequence, which has been ligated into the vector to recirculize the plasmid. For use in recombination experiments large-scale plasmid DNA preparations were linearized by *Mlu* I digestion and can be recombined with PCR amplified *gag*-genes derived from the HIV strains selected in the presence of virus cell entry inhibitors.

Construction of the gag/gp160-deleted clone.

The described *gp160*-deleted clone has been digested with the restriction enzymes *BssH* II and *Spe* I to generate the *p17/gp160*-deleted clone (Figure 5a). The linker sequence as described above in the construction of the *gag*-deleted clone, was ligated into the vector to recirculize the *p17/gp160*-deleted plasmid. For use in recombination experiments large-scale plasmid DNA preparations of the *p17/gp160*-deleted clone were linearized in parallel. One batch was digested with *Mlu* I at the *gag*-deletion, whereas another batch was digested with *Sal* I at the *env*-deletion. These linearized plasmids can be co-transfected in the appropriate cell line with the PCR-derived *gag* and *env* sequences derived from the HIV strains selected in the presence of virus cell entry inhibitors (Figure 5b).

Due to the low percentage of occurrence of homologous recombination after electroporation, tranfection of cells by means of lipofection may be a better way of introducing DNA in the target cells. Since, 293 cells are known to have a high tranfection efficiency, these cells can be used for lipofection, when MT-4 or other target cells for HIV are added after this tranfection.

Generation of the linker sequence for the p17- and p17/gp160-deleted clone

A 126 nucleotide base pair fragment of pNL4.3 was amplified by PCR using Expand ™ High Fidelity PCR system. The PCR reaction was performed on a Biometra Trioblock using the primers AV14 (5'-CTC TCT CGA CGC AGG ACT CGG CTT GCT GAA-3') and PC-VAB (5' ACT AGT AGT TCC TGC TAT GTC ACG CGT CTC TCT CCT TCT AGC C 3'). PC-VAB was specifically designed to insert the *Mlu* I and *Spe* I restriction sites in the linker sequence. Therefore, a tail with the required sequence was added to the obligatory sequence to amplify the fragment in front of the start codon of *p17*, i.e. the packaging signal (Figure 6). The cycling conditions were as follows: 40 cycles consisting of 15 sec 95°C, 30 sec 55°C and 30 sec 68°C. A final extension was performed at 72°C for 10 min.

The resulting PCR fragment was digested with the restriction endonucleases BssH II and Spe I and subsequently purified by phenol/chloroform extraction.

Example 5.: Co-transfection of the different sequences derived from the wild-type and resistant strains with their corresponding delected clone.

MT-4 cells were subcultured at a density of 500.000 cells/ml on the day before transfection. Cells were pelleted and resuspended in phosphate-buffered saline at a concentration of 3.125 $x10^6$ cells/ml. For each transfection 2.5 $x10^6$ cells (0.8ml) were used. Transfections were performed by electroporation using an EASYJECT (Eurogentec, Seraing, Belgium) and electroporation cuvettes (Eurogentec, Seraing, Belgium). For *gp120* recombination experiments, MT-4 cells were cotransfected with 10 µg of the linearized *gp120*-deleted NL4.3 clone and 2 µg (3 tubes of 50 µl) purified and concentrated AV312-AV313 inner PCR product (PCR Purification Kit, Qiagen, Westburg, Leusden, The Netherlands). For *gp41* recombination experiments, cells were cotransfected with 10 µg of the linearized *gp41*-deleted NL4.3 clone and 2 µg purified and concentrated AV320-AV321 PCR product, whereas the AV310-AV319 PCR product was co-transfected with the *gp160*-deleted clone in *gp160* recombination experiments. In the *integrase* recombination assay, MW3-MW4 PCR products were co-transfected with the *integrase*-deleted clone. The electroporation conditions were 300 µF and 300 V for all transfections. After 30 minutes incubation at room temperature, the transfected cell suspension in 5 ml of culture medium was incubated at 37°C in a humidified atmosphere with 5% CO_2. The recombinant virus was harvested by centrifugation, when full CPE was observed in the culture (about 6 days post-transfection) and stored in 1 ml aliquots at -80°C for subsequent infectivity and drug susceptibility determinations (IC_{50}) in the MT-4/MTT assay.

DISCUSSION

In view of the strong correlation between the appearance of drug-resistant strains and therapeutic failure, the availibility of new drugs effective at new targets within the HIV replication cycle (other than RT and PRO) and thus being effective against the current drug-resistant viruses should allow more potent combination strategies (Larder *et al.*, 1998, Vandamme *et al.*, 1999a). An attractive and promising new target is virus-cell binding and virus-cell fusion. Several compounds inhibiting viral entry into cells have been described. Another interesting target for new inhibitors is the viral enzyme integrase, since integration is an essential step for HIV replication and since no human homologue is known to play a role during normal cell metabolism. Still, only one authentic integrase inhibitor in cell culture has so far been reported (Hazuda *et al.*, 2000).

Since inhibitors of viral entry, i.e. AMD3100 and T-20 are already subject of clinical phase II studies, the development of phenotypic drug resistance assays, that can measure the sensitivity of patient isolates towards these new compounds, is mandatory. Knowledge on development of HIV-1 resistance against entry inhibitors in cell culture will help the interpretation of clinical data.

Here, we describe the construction of *gp120*-, *gp41*- and *gp160* recombination assays and the application on several drug-resistant HIV-1 strains selected *in vitro* in the presence of entry inhibitors. In addition, we describe the development of an integrase recombination assay.

The study of resistance development towards compounds inhibiting viral entry can contribute to the elucidation of their molecular mode of action. Drug-resistant HIV-1(NL4.3) strains were selected *in vitro* in the presence of increasing concentrations of the entry inhibitors L-CA (L-CA^{res}), BRI2923 (BRI2923^{res}) and AMD3100 (AMD3100^{res}) (Table 1). Mutations in the *gp120* genes of these *in vitro* selected strains were detected by sequencing analysis (Table 5). Moreover, the strain BRI2923^{res} contained mutations in the *gp41*-gene (Table 6). Antiviral drug susceptibility assays, such as the MT-4/MTT assay, allow the investigation of resistance and cross-resistance patterns of these mutant strains to various anti-HIV drugs by determining their 50% inhibitory concentrations (IC_{50}) (Pauwels *et al.*, 1988, Vandamme *et al.*, 1999b) (Table 7).

The *env*-recombination techniques described, wherein *gp120*-, *gp41*- and *gp160*- coding sequences can be recombined into the genetic background of a proviral clone deleted for the corresponding gene, are helpful to further delineate to what extent some regions are responsible for the observed resistance of the selected resistant strains. These different *env*-recombination assays were applied on HIV-1(NL4.3) virus strains selected in the presence of L-CA, BRI2923 and AMD3100. Comparison of the degree of resistance of the *in vitro* selected strain with the corresponding recombinant virus strain may be considered as indicative for the role of the recombined region in the observed phenotypic resistance (Tables 8-10).

Recombination of *gp120* (and *gp160*) fully rescued the phenotypic resistance of the virus strain selected in the presence of L-CA. L-CA was originally proffered as an integrase inhibitor, because of its inhibitory activity in an oligonucleotide-based integrase assay (McDougall *et al.*, 1998). However, in cell culture this compound was found to inhibit HIV replication by interfering with viral entry (Pluymers *et al.*, 2000). This finding was corroborated by the appearance of mutations in *gp120,* and not in *integrase,* in the viral strain selected in the presence of this compound. *gp120*-recombination in a wild-type background resulted in a virus displaying the same resistance profile as the *in vitro* selected strain.

Since the BRI2923^{res} *gp120*- and *gp160*- recombined strains displayed wild-type sensitivity towards the compound BRI2923, it appears that resistance against BRI2923 is attributed to mutations in other genes distinct from the genes encoding the envelope glycoproteins. The close proximity of the matrix protein MA (p17) to the lipid bilayer supports the idea that a direct interaction takes place between the endodomain of the glycoprotein gp41 and the matrix domain of gag (Freed *et al.*, 1998). Therefore, we have looked for mutations in the gag encoding sequences of the BRI2923^{res} strain. Mutations were found in the basic domain, that is known to play a role in membrane binding (Zhou *et al.*, 1994), in the p17 encoding part of the gag gene of BRI2923^{res} and not in the NL4.3^{WT}, L-CA^{res} and AMD3100^{res} strains (Table 11).

To further asses the impact of these mutations on the observed resistance of the BRI2923^{res} strain, recombination assays have been developed, wherein the p17 and a small part of the p24 encoding part of the *gag* gene by itself and in combination with the *env*-region can be placed in a proviral clone. To construct the double deleted clone, the p17/p24 encoding

part of the *gag* gene has been excised out the *gp160*-deleted clone. The resulting clone has then been linearized at the position of the *gag*-deletion and in parallel at the position of the *env*-deletion. These linearized plasmids were co-transfected with the PCR-derived *gag* and *env* sequences. The *gag* sequences recombine in the plasmid linearized at the *gag*-deletion, whereas the *env* sequences recombine in the plasmid linearized at the *env*-deletion. Viable virus particles will only be generated by an additional recombination step during virus replication, when one RNA strand of the HIV virion contains the *gag*-recombined plasmid and the other RNA strand contains the *env*-recombined plasmid. Moreover, one has to take care that the packaging signal situated upstream from the *gag* sequences is not disrupted by endonuclease digestion, required to cut out the p17/p24 encoding region. Since the start codon of the gag polyprotein is located directly behind the end of the packaging signal, we used a unique restriction site located in the packaging signal. The double deleted vector has been completed via the reintroduction of the packaging signal by means of designing a linker sequence that contains the rest of the packaging signal. By generating a novel unique restriction site in this linker sequence at the start codon of the gag polyprotein, the resulting deleted clone can be linearized without disturbing the packaging signal. The same primers and linker sequences have been used to develop the recombination assay for the p17/p24 encoding region solely. In this case however, the disruption of the packaging signal does not affect recombination, since no additional recombination step during viral replication is required.

Since the CXCR4 antagonist AMD3100 and the fusion inhibitor T-20 have proceeded to clinical Phase II trials, we assessed the use of these recombination assays for phenotypic evaluation of clinical isolates that may have developed resistance at the level of the envelope glycoproteins. Since complete resistance of the AMD3100 resistant virus strain could be fully rescued by *gp120*-recombination, we conclude that *env*-recombination is a powerful diagnostic tool to assess the susceptibility/resistance profile of clinical isolates to AMD3100. When applied on clinical isolates, *gp160*-recombination will be more complete than that of *gp120*.

Because resistance towards the fusion inhibitor T-20 is reflected by mutations in the *gp41* gene (Rimsky *et al.*, 1998), the *gp41*- or *gp160*-recombination assay can be used for susceptibility testing of clinical isolates of patients under treatment with this compound. The mutations in the *gp41* gene of the BRI2923^{res} strain were responsible for the observed cross-resistance of the BRI2923^{res} strain against T-20, since *gp41*-recombination of the

BRI2923^{res} strain resulted in a virus with the same sensitivity towards T-20 as the *in vitro* selected and the BRI2923^{res} *gp160*-recombined strain.

The wild-type *gp120*-recombined strain, as well as the L-CA^{res} *gp120*-recombined and AMD3100^{res} *gp120*-recombined viruses, showed a 5- to 10-fold lower sensitivity to the inhibitory effect of T-20 in comparison to the NL4.3 wild-type and the L-CA^{res} and AMD3100^{res} *in vitro* selected strains. Since this is the case for all *gp120*-recombined viruses. (with the exception of the BRI2923^{res} *gp120*-recombined strain), the reduced sensitivity can be explained by the genetic background of the *gp120*-deleted clone, probable the *gp41* gene of the plasmid pNL4.3. Indeed, the strain obtained directly after transfection of MT-4 cells with the plasmid pNL4.3 showed a 10-times lower susceptibility to T-20 in comparison with the NL4.3 wild-type strain that is adapted to cell culture and was used for the selection (data not shown).

Besides *env*-recombinations, we introduced the integrase gene of a HIV-1(III_B) strain in the integrase-deleted proviral pNL4.3 clone. Viable virus was obtained and evaluauted The resulting virus showed the same sensitivity for several compounds [i.e. zidovudine, nevirapine, ritonavir and diketo acid (L-708,988)] compared to the HIV-1(III_B) and HIV-1(NL4.3) strain (Table 12). Homologeous recombination was demonstrated by sequencing analysis, providing a proof-of-principle regarding the integrase-recombination assay.

The *env*- and *integrase* recombination assays are research tools for the study of the mode of action of new compounds inhibiting HIV entry or integration. These recombination assays delineate the region(s) responsible for the observed phenotypic resistance of a particular drug-resistant HIV strain.

In addition, the recombination technique described here could also be used to investigate the unknown mechanism of action of new drugs. A battery of gene-recombinations, recombining each gene separately should greatly facilitate those mechanism of action studies. We have demonstrated for the first time the design and the application of the *gp120-*, *gp41-*, *gp160-* and *integrase*-recombination assays. This technique can additionally be extended to other genes of the HIV genome encoding proteins that will act as targets of antiviral therapy *e.g.* the matrix protein (MA, p17), capsid antigen (CA, p24), nucleocapsid (NC, p7), regulatory proteins, etc. Moreover, the recombination methods, mentioned above, can as well be designed for viruses other than HIV.

In conclusion, we used chimeric virus technology for *env* as a research tool to prove that L-CA exclusively targets gp120 or the interaction of gp120 with a cellular receptor. We also showed that resistance against BRI2923 involves another gene, namely MA (p17). Furthermore, since the resistance of the AMD3100 resistant virus strain can be completely reproduced by *gp120*-recombination, gp120 may be considered as the sole viral (glyco)protein involved in the anti-HIV action of this compound. The *env*-recombination technique may be a powerful diagnostic tool to assess the susceptibility of clinical isolates to AMD3100.

Table 1. *In vitro* selection of HIV-1(NL4.3) strains resistant to virus cell entry inhibitors

	L-CA^{res}	BRI2923^{res}	AMD3100^{res}
Final concentration of the compound used for selection (μg/ml)	30 (4)[a]	20 (67)	500 (125.000)
Number of passages	23	30	63

[a] fold increase in IC_{50} against wild-type HIV-1(NL4.3)

Table 2. Optimizing the different *env* PCR's

gene	length fragment	position of NL4.3	primers	program
gp120 (the last 35 aa excluded)	2212 bp	5447-7659	OUTER AV310 (5'-AGC AGG ACA TAA T/CAA GGT AGG-3') corresponding to position 5447-5467 of NL4.3 AV311 (5' GGA GAA GTG AAT TAT ATA AG/AT ATA AAG TAG-3') corresponding to position 7630-7659 of NL4.3	denaturation step of 3 min. at 95°C followed by 40 cycles consisting of 45 sec 95°C, 30 sec 50°C, 2 min. 72°C final extension 10 min. at 72°C
	2105 bp	5549-7629	INNER AV312 (5'-AGA A/GGA C/TAG ATG GAA CAA GCC CCA G-3') corresponding to position 5549-5573 of NL4.3 AV313 (5'-GAC CTG GAG GAG GAA/G ATA TGA G/AGG A-3') corresponding to position 7605-7629 of NL4.3	denaturation step of 3 min. at 95°C 30 cycles consisting of 45 sec 95°C, 30 sec 58°C, 2 min. 72°C final extension 10 min. at 72°C
	Sequencing using the 6 internal primers AV304, AV305, AV306, AV307, AV308, AV309 and the PCR primer AV313			
gp41 (the last 35 aa of *gp120* included)	1560 bp	7353-8913	AV320 (5'-ATT GTA/G GAG GA/GG AAT TTT TCT ACT G-3') corresponding to position 7353-7378 of NL4.3 AV321 (5' TTG CTA/G CTT GTG ATT GCT/C CCA TG-3') corresponding to position 8890-8913 of NL4.3	denaturation step of 2 min. at 95°C 40 cycles consisting of 15 sec 95°C, 30 sec 55°C, 2 min. 68°C final extension 10 min. at 72°C
	Sequencing using the 10 internal primers AV322, AV323 AV324, AV325, AV326, AV327, AV328, AV329, AV330 and AV331			
gp160	3577 bp	5549-9024	AV310 (5'-AGC AGG ACA TAA T/CAA GGT AGG-3') corresponding to position 5447-5467 of NL4.3 AV319 (5'-GCT G/C CC TTA/G TAA GTC ATT GGT CT-3') corresponding to position 9001-9024 of NL4.3	denaturation step of 2 min. at 95°C 40 cycles consisting of 15 sec 95°C, 30 sec 55°C, 4 min. 68°C final extension 10 min. at 72°C

Table 3. Optimizing the *gag* PCR

gene	length fragment	position of NL4.3	primers	program
p17+ p24	1424 bp	59-1483	AV14 (5'-CTC TCT CGA CGC AGG ACT CGG CTT GCT GAA-3') corresponding to position 59-88 of NL4.3 BVR2 (5'-GCC AG/AA TC/TT TCC CTA AAA AAT TAG CC-3') corresponding to position 1457-1483 of NL4.3	denaturation step of 2 min. at 95°C 40 cycles consisting of 15 sec 95°C, 30 sec 55°C, 90 sec. 72°C final extension 10 min. at 72°C
	Sequencing using the 11 internal primers AV13 (5'-CTG CGA ATC GTT CTA GCT CCC TGC TTG CCC -3') corresponding to position 245-274 of NL4.3, AV26 (5'-GCT ATG TCA CTT CCC CTT GGT TCT C -3') corresponding to position 829-853 of NL4.3, AV54 (5'-GAG ACC ATC AAT GAG GAA GCT GC-3') corresponding to position 774-796 of NL4.3, AV58, (5'-GGC TAA TTT TTT AGG-3') corresponding to position 1457-1471 of NL4.3, AV159 (5'-GGG GTT AAA TAA AAT AGT AAG-3') corresponding to position 971-991 of NL4.3, AV103 (5'-GCC ATA TCA CCT AGA ACT TT-3') corresponding to position 603-622 of NL4.3, GAG-1 (5'-AGA AAC ATC AGA AGG CTG TAG-3') corresponding to position 320-340 of NL4.3, BVR3 (5'-TTT CCA ACA GCC CTT TTT CCT AG-3') corresponding to position 1368-1390 of NL4.3, BVR4 (5'-AGA CAC CAA G/AGA AGC-3') corresponding to position 352-366 of NL4.3, BVR5 (5'-TCC T/CTC TGA TAA TGC TG-3') corresponding to position 669-685 of NL4.3 and BVR6 (5'-TAG AAG AAA TGA TGA CAG C-3') corresponding to position 1195-1213 of NL4.3	

Table 4. Optimizing the *integrase* PCR

gene	length fragment	position of NL4.3	primers	program
integrase	2578 bp	3117-5546	MW3 (5'-TAT GTA GGA/G TCT GAC/T TTA GAA ATA GGG-3') corresponding to position 3117-3144 of NL4.3 MW4 (5'-TAA CAC TAG GCA A/GAG GTG GCT T-3') corresponding to position 5524-5546 of NL4.3	denaturation step of 2 min. at 95°C 40 cycles consisting of 15 sec 95°C, 30 sec 60°C, 3 min. 68°C final extension 10 min. at 72°C
	Sequencing using the 8 internal primers: IN-PCRA (5'-GGAGGAAATGAACAAGTAGAT-3' corresponding to position 4181-4202 of NL4.3), HP4392C (5'-TCTACTTGTCCATGCATGGCTTC-3' corresponding to position 4377-4399 of NL4.3), IN-SEQ1 (5'-TTAAGATGTTCAGCCTGATCT-3' corresponding to position 4733-4753 of NL4.3), IN-SEQ2 (5'-CATTGTCTGTATGTACTGTTT-3' corresponding to position 4567-4587 of NL4.3), IN-SEQ3 (5'-GGATATATAGAAGCAGAAGTAA-3' corresponding to position 4479-4500 of NL4.3), IN-SEQ4 (5'-GAACATCTTAAGACAGCAGTA-3' corresponding to position 4743-4753 of NL4.3), IN-SEQ5 (5'-AAGCTCCTCTGGAAAGGTGAA-3' corresponding to position 4953-4973 of NL4.3), and IN-PCRB (5'-CCTTGAAATATACATATGGTG-3' corresponding to position 5125-5145 of NL4.3)			

Table 5. Genotypic analysis of the *gp120* genes of drug-resistant HIV-1(NL4.3) strains

Region	V2			V3								
Amino acid	147	154	160	270	272	274	276	280	290	292	295	299
NL4.3^{WT}	F	V	S	N	N	R	S	Q	I	K	N	A
L-CA^{res}	.	.	N	.	.	.	.	.	.	.	D	.
BRI2923^{res}	L	E	.	.	.	.	.	.	.	Q	.	.
AMD3100^{res}	L	.	.	+Y	S	T	R	H	V	.	H	T

Region	C3	V4									C4			V5	C5
Amino acid	323	366	367	368	369	370	372	378	379	387	389	393	412	435	459
NL4.3^{WT}	F	F	N	S	T	W	T	T	E	P	R	F	Q	S	V
L-CA^{res}	.	.	.	.	.	.	N	N	Q	.	.	.	.	.	.
BRI2923^{res}	Y	_	_	_	_	_	.	.	.	.	E	V	.	.	.
AMD3100^{res}	.	_	_	_	_	_	.	.	.	L	.	.	E	P	I

Table 6. Genotypic analysis of the *gp41* genes of drug-resistant HIV-1(NL4.3) strains

position of NL4.3	2	22	33	49	126	210	213	216	308
NL4.3 ^{WT}	V	A	L	E	N	L	P	P	A
BRI2923^{res}	.	V	S	.	.	.	.	P/L	A/T

One letter code for the amino acids

Alanine	A
Arginine	R
Asparagine	N
Aspartic acid	D
Cysteine	C
Glutamine	Q
Glutamic acid	E
Glycine	G
Histidine	H
Isoleucine	I
Leucine	L
Lysine	K
Methionine	M
Phenylalanine	F
Proline	P
Serine	S
Threonine	T
Tryptophane	W
Tyrosine	Y
Valine	V

Clarification of the symbols

. no change in amino acid

+ insertion of an amino acid

- deletion of an amino acid

Table 7. Drug-susceptibility of HIV-1(NL4.3)-derived strains that were in vitro *selected in the presence of virus cell entry inhibitors in comparison tot the wild-type HIV-1(NL4.3) strain*

	IC_{50} [a] (fold resistance) [b]			
Virus strains	L-CA	BRI2923	AMD3100	T-20
$NL4.3^{WT}$	5.6±3.5	0.29±0.22	0.012±0.005	0.029±0.012
NL4.3/L-$CA^{res\ c}$	**31.7±2.6 (5.7)**	1.00±0.11 (3.4)	0.014±0.003 (1.2)	0.042±0.014 (1.4)
NL4.3/$BRI2923^{res\ d}$	14.8 (2.6)	**3.4±2.0 (11.7)**	0.032±0.030 (2.7)	2.17±0.82 (74.8)
NL4.3/$AMD3100^{res\ e}$	6.1 (1.1)	5.1±3.3 (17.6)	**1.9±1.4 (158.3)**	0.080±0.034 (2.8)

[a] **50% inhibitory concentration (IC_{50}) (in μg/ml), or concentration of the compound required to inhibit the cytopathic effect of the different HIV strains by 50% in MT-4 cells.**

[b] ratio of the IC_{50} of the compound against the *in vitro* selected HIV-1(NL4.3)-derived strain to the IC_{50} of the compound against the HIV-1(NL4.3) wild-type strain.

[c] HIV-1(NL4.3)-derived *in vitro* selected resistant (^{res}) strain in the presence of L-CA.

[d] HIV-1(NL4.3)-derived *in vitro* selected resistant (^{res}) strain in the presence of BRI2923.

[e] HIV-1(NL4.3)-derived *in vitro* selected resistant (^{res}) strain in the presence of AMD3100.

Table 8. Drug-sensitivity of the *gp120*-recombined viruses derived from the HIV-1(NL4.3)-derived strains that were selected *in vitro* in the presence of virus cell entry inhibitors

Virus strains	IC_{50} [a] (fold resistance) [b]			
	L-CA	BRI2923	AMD3100	T-20
R120/NL4.3[WT]	6.8±1.7	0.21±0.11	0.0093±0.0066	0.15±0.01
R120/L-CA[res c]	**30.1±20.3 (4.4)**	0.86±0.30 (4.1)	0.016±0.006 (1.7)	0.20±0.04 (1.3)
R120/BRI2923[res d]	13.4 (2.0)	**0.43±0.27 (2.0)**	0.0062±0.0052 (0.7)	0.24±0.14 (1.6)
R120/AMD3100[res e]	5.0±1.6 (0.7)	4.2±2.3 (20)	**0.65±0.43 (69.9)**	0.43±0.28 (2.9)

[a] **50% inhibitory concentration (IC_{50}) (in μg/ml), or concentration of the compound required to inhibit the cytopathic effect of different HIV strains by 50% in MT-4 cells.**

[b] ratio of the IC_{50} of the compound against the *gp120*-recombined *in vitro* selected strain to the IC_{50} of the compound against the HIV-1(NL4.3) *gp120*-recombined wild-type strain.

[c] HIV-1(NL4.3) wild-type strain recombined with the *gp120* gene of the *in vitro* selected resistant ([res]) strain in the presence of L-CA.

[d] HIV-1(NL4.3) wild-type strain recombined with the *gp120* gene of the *in vitro* selected resistant ([res]) strain in the presence of BRI2923.

[e] HIV-1(NL4.3) wild-type strain recombined with the *gp120* gene of the *in vitro* selected resistant ([res]) strain in the presence of AMD3100.

Table 9. Drug-sensitivity of the *gp41*-recombined viruses derived from the HIV-1(NL4.3)-derived strains that were selected *in vitro* in the presence of entry inhibitors

Virus strains	IC_{50} [a] (fold resistance) [b]			
	L-CA	BRI2923	AMD3100	T-20
R41/NL4.3[WT]	1.53	0.20±0.09	0.0032±0.0002	0.025±0.004
R41/L-CA[res c]	**1.83** **(1.2)**	0.19±0.05 (1.0)	0.0032±0.0006 (1.0)	0.025±0.005 (1.0)
R41/BRI2923[res d]	0.73 (0.5)	**0.39** **(2.0)**	0.0042 (1.3)	0.61±0.06 (24.4)
R41/AMD3100[res e]	2.43 (1.6)	0.34±0.24 (1.7)	**0.0033±0.0019** **(1.0)**	0.017±0.016 (0.7)

[a] 50% inhibitory concentration (IC_{50}) (in µg/ml), or concentration of the compound required to inhibit the cytopathic effect of different HIV strains by 50% in MT-4 cells.

[b] ratio of the IC_{50} of the compound against the *gp41*-recombined *in vitro* selected strain to the IC_{50} of the compound against the HIV-1(NL4.3) *gp41*-recombined wild-type strain.

[c] HIV-1(NL4.3) wild-type strain recombined with the *gp41* gene of the *in vitro* selected resistant ([res]) strain in the presence of L-CA.

[d] HIV-1(NL4.3) wild-type strain recombined with the *gp41* gene of the *in vitro* selected resistant ([res]) strain in the presence of BRI2923.

[e] HIV-1(NL4.3) wild-type strain recombined with the *gp41* gene of the *in vitro* selected resistant ([res]) strain in the presence of AMD3100.

Table 10. Drug-sensitivity of the *gp160*-recombined viruses derived from the HIV-1(NL4.3)-derived strains that were selected *in vitro* in the presence of entry inhibitors

	IC_{50} [a] (fold resistance) [b]			
Virus strains	L-CA	BRI2923	AMD3100	T-20
R160/NL4.3^{WT}	2.4	0.23±0.13	0.0054±0.0022	0.029±0.0039
R160/BRI2923$^{res\ c}$	6.7 (2.8)	**0.6±0.01** **(2.6)**	0.0083±0.0067 (1.5)	0.70±0.17 (24.1)
R160/AMD3100$^{res\ d}$	3.6 (1.5)	3.13±0.73 (13.6)	**0.74±0.24** **(137)**	0.03±0.0001 (1.0)

[a] 50% inhibitory concentration (IC_{50}) (in μg/ml) or concentration of the compound required to inhibit the cytopathic effect of different HIV strains by 50% in MT-4 cells.

[b] ratio of the IC_{50} of the compound against the *gp160*-recombined *in vitro* selected strain to the IC_{50} of the compound against the HIV-1(NL4.3) *gp160*-recombined wild-type strain.

[c] HIV-1(NL4.3) wild-type strain recombined with the *gp160* gene of the *in vitro* selected resistant (^{res}) strain in the presence of BRI2923.

[d] HIV-1(NL4.3) wild-type strain recombined with the *gp160* gene of the *in vitro* selected resistant (^{res}) strain in the presence of AMD3100.

Table 11. Genotypic analysis of the *gag* genes of drug-resistant HIV-1(NL4.3) strains

position of NL4.3	12	27	35	62	125
NL4.3^{WT}	E	K	V	G	A
BRI2923^{res}	K	R	I	R	A/E

Table 12. Susceptibility of the HIV-1(III_B) integrase-recombined HIV-1(NL4.3) strain in comparison to the HIV-1(III_B) and HIV-1(NL4.3) strains

compound	class of compound	R IN/III_B[a]	HIV-1(III_B)	HIV-1(NL4.3)
zidovudine (AZT)	NRTI[b]	0.0012±0.001	0.0011±0.001	0.0010±0.002
nevirapine (BOE/BIRG587)	NNRTI[c]	0.029±0.007	0.019±0.005	0.020±0.006
ritonavir (ABT/A84538)	PROI[d]	0.049±0.004	0.089±0.006	0.060±0.005
diketo acid (L-708,988)	INI[e]	5.5±0.5	5.0±0.4	10.0±0.8

[a] HIV-1(III_B) integrase-recombined HIV-1(NL4.3) strain
[b] Nucleoside reverse transcriptase inhibitor
[c] Non-nucleoside reverse transcriptase inhibitor
[d] Protease inhibitor
[e] Integrase inhibitor

CITED LITERATURE

Adachi, A., Gendelman, H.E., Koenig, S., Folks, T., Willey, R., Rabson, A., Martin, M.A. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. *J. Virol.* 1986; *59*: 284-291

Baba, M., Pauwels, R., Balzarini, J., Arnout, J., Desmyter, J., De Clercq, E. Mechanism of inhibitory effect of dextran sulfate and heparin on replication of human immunodeficiency virus in vitro. *Proc. Natl. Acad. Sci. USA* 1988; *85 (16)*: 6132-6136

Bridger, G.J., Skerlj, R.T., Padmanabhan, S., Martelucci, S.A., Henson, G.W., Abrams, M.J., Joa, H.C., Witvrouw, M., De Vreese, K., Pauwels, R. and De Clercq, E. Synthesis and structure-activity relationship of phenylenebis(methylene)-linked bis-tetraazamacrocycles that inhibit HIV replication. Effect of heteroaromatic linkers on the activity of bicyclams. *J. Med. Chem.* 1995; *38*: 366-378

Cheng-Mayer, C., Brown, A., Harouse, J., Luciw, P.A., Mayer, A.J. Selection for neutralization resistance of the simian/human immunodeficiency virus SHIV $_{SF33A}$ variant in vivo by virtue of sequence changes in the extracellular envelope glycoprotein that modify N-linked glycosylation. *J. Virol.* 1999; *(73)7*: 5294-5300

De Clercq, E., Yamamoto, N., Pauwels, R., Baba, M., Schols, D., Nakashima, H., Balzarini, J., Debyser, Z., Murrer, B.A., Schwartz, D., *et al.* Potent and selective inhibition of human immunodeficiency virus (HIV)-1 and HIV-2 replication by a class of bicyclams interacting with a viral uncoating event. *Proc. Natl. Acad. Sci. USA* 1992; *89*: 5286-5290

De Clercq, E., Yamamoto, N., Pauwels, R., Balzarini, J., Witvrouw, M., De Vreese, K., Debyser, Z., Rosenwirth, B., Peichl, P., Datema, R. *et al.* Highly potent and selective inhibition of human immunodeficiency virus by the bicyclam derivative JM3100. *Antimicrob. Agents Chemother.* 1994; *38*: 668-674.

De Vreese, K., Kofler-Mongold, V., Leutgeb, C., Weber, V., Vermeire, K., Schacht, S., Anné, J., De Clercq, E., Datema, R., Werner, G. The molecular target of bicyclams, potent inhibitors of human immunodeficiency virus replication. *J. Virol.* 1996a; *70*: 689-696

De Vreese, K., Reymen, D., Griffin, P., Steinkasserer, A., Werner, G., Bridger, G.J., Esté, J., James, W., Henson, G.W., Desmyter, J., Anné, J., De Clercq, E. The bicyclams, a new class of potent human immunodeficiency virus inhibitors, block viral entry after binding. *Antiviral. Res.* 1996b; *29*: 209-219

Drucker, J. Development and Clinical Evaluation of T-20: An inhibitor of HIV Membrane Fusion. 13th International Conference on Antiviral Research, poster 161, 2000, Baltimore, Maryland

Esté, J.A., Schols, D., De Vreese, K., Van Laethem, K., Vandamme, A.M., Desmyter, J., De Clercq, E. Development of resistance of human immunodeficiency virus type 1 to dextran sulfate associated with the emergence of specific mutations in the envelope gp120 glycoprotein. *Mol. Pharmacol.* 1997; *52*: 98-104

Esté, J.A., Cabrera, C., Blanco, J., Gutierrez, A., Bridger, G., Henson, G., Clotet, B., De Clercq, E. Shift of clinical human immunodeficiency virus type 1 isolates from X4 to R5 and prevention of emergence of the syncytium-inducing phenotype by blockade of CXCR4. *J. Virol.* 1999; *73*:5577-5585

Freed, E.O. HIV-1 Gag proteins: diverse functions in the virus life cycle. *Virology* 1998; *251*: 1-15

Hazuda, D.J., Felock, P., Witmer, M., Wolfe, A., Stillmock, K., Grobler, J.A., Espeseth, A., Gabryelski, L., Schleif, W., Blau, C., Miller, M.D. Inhibitors of strand transfer that prevent integration and inhibit HIV-1 replication in cells. *Science* 2000; *287*: 646-650

Hendrix, C., Flexner, C., MacFarland, R., Giandomenico, C., Schweitzer, Henson, G. Chemokine CXCR-4 receptor blocker, AMD3100: Clinical and pre-clinical studies of

pharmacokinetics and safety. 6th Conference on Retroviruses and opportunistic infections 1999, Chicago, Illinois

Hertogs, K., de Béthune, M.-B., Miller, V., Ivens, T., Schel, P., Van Cauwenberge, A., Van Den Eynde, C., Van Gerwen, V., Azijn, H., Van Houtte, M., Peeters, F., Staszewski, S., Conant, M., Bloor, S., Kemp, S., Larder, B., Pauwels, R. A rapid method for simultaneous detection of phenotypic resistance to inhibitors of protease and reversre transcriptase in recombinant human immunodeficiency virus type 1 isolates from patients treated with antiviral drugs. *Antimicrob. Agents Chemother.* 1998; *42 (2)*: 269-276

Kellam, P., Larder, B.A. Recombinant Virus Assay: a rapid, phenotypic assay for assessment of drug susceptibility of human immunodeficiency virus type 1 isolates. *Antimicrob. Agents. Chemother.* 1994; *38:* 23-30

Kilby, J.M., Hopkins, S., Venetta, T.M., Di Massimo, B., Cloud, G.A., Lee, J.Y., Alldredge, L, Hunter, E., Lambert, D., Bolognesi, D., Matthews, T., Johnson, M.R., Nowak, M.A., Shaw, G.M., Saag, M.S. Potent suppression of HIV-1 replication in humans by T-20, a peptide inhibitor of gp41-mediated virus entry. *Nature Med.* 1998; *4*: 1302-1307

Kuritzkes, D.R. Drug resistance testing: Time to be used in clinical practice ? *AIDS Rev* 1999; *1*: 45-50

Larder, B., Richman, D., Vella, S. HIV resistance and implications for therapy. Atlanta (GA): Medicom Inc., 1998

Maschera, B., Furfine, E., Blair, E.D. Analysis of resistance to human immunodefiency virus type 1 protease inhibitors by using matched bacterial expression and proviral infection vectors. *J. Virol.* 1995; *69*: 5431-5436

McDougall, B., King, P.J., Wu, B.W., Hostomsky, Z., Reinecke, M.G., Robinson, W.E. Jr. Dicaffeoylquinic and dicaffeoyltartaric acids are selective inhibitors of human immunodeficiency virus type 1 integrase. *Antimicrob. Agents Chemother.* 1998; *42*: 140-146

Miyoshi, I., Taguchi, H., Kobonishi, I., Yoshimoto, S., Ohtsuki, Y., Akagi, T. Type C virus-producing cell lines derived from adult T cell leukemia. *Gann. Monogr.* 1982; *28*: 219-228

Pauwels, R., Balzarini, J. Baba, M. Snoeck, R., Schols D., Herdewijn P., Desmyter J., De Clercq, E.. Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. *J. Virol. Methods* 1988; *20*: 309-321

Pluymers, W., Neamati, N., Pannecouque, C., Fikkert, V., Marchand, C., Burke, T.R. Jr., Pommier, Y., Schols, D., De Clercq, E., Debyser, Z., Witvrouw, M. Viral entry as the primary target of anti-HIV activity of chicoric acid and its tetra-acetyl esters. *Mol. Pharmacol.* 2000; *58*: 641-648

Popovic, M., Sarngadharan, M.G., Read, E., Gallo, R.C. Detection, isolation and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. *Science (Wash DC)* 1984; *224*: 497-500

Rimsky, L.T., Shugars, D.C., Matthews T.J. Determinants of human immunodeficiency virus type 1 resistance to gp41-derived inhibitory peptides. *J. Virol.* 1998; *72*: 986-993

Schols, D., Struyf, S., Van Damme, J., Esté, J.A., Henson, G., De Clercq, E. Inhibition of T-tropic HIV strains by selective antagonization of the chemokine receptor CXCR4. *J. Exp. Med.* 1997; *186*: 1393-1388

Vandamme, A.-M., Van Laethem, K., De Clercq, E. Managing Resistance to anti-HIV drugs. An important consideration for effective disease management. *Drugs* 1999a; *57 (3)*: 337-361

Vandamme, A.-M., Witvrouw, M., Pannecouque, C., Balzarini, J., Van Laethem, K., Schmit, J.-C., Desmyter, J., and De Clercq, E. "Evaluating clinical isolates for their phenotypic and genotypic resistance against anti-HIV drugs" in "Methods in Molecular Medicine 1999b, vol. 24: Antiviral Methods and Protocols": Kinchington, D. and Schinazi, R.F.; Humana Press Inc., Totowa, NJ

Vartanian, J.P. AMD3100 AnorMED. *IDrugs* 2000; *3(7)*: 811-816

Verrier, F., Borman, A.M., Brand, D. and Girard, M. Role of the HIV type 1 glycoprotein 120 V3 loop in determining coreceptor usage. *AIDS Res. Hum. Retroviruses* 1999; *15-(8)*: 731-743

Witvrouw, M., Fikkert, V., Pluymers, W., Matthews, B, Schols, D., Raff, J., Debyser, Z., De Clercq, E., Holan, G., Pannecouque, C. Polyanionic (i.e. Polysulfonate) dendrimers can inhibit the replication cycle of human immunodeficiency virus (HIV) by interfering with both virus absorption and later steps (reverse transcriptase/integrase) in the virus replicative cycle. *Mol. Pharmacol.* 2000; *58*: 1100-1108

Zhao, H. and Burke, T.R.Jr. Facile syntheses of (2R,3R)-(-)- and (2S,3S)-(+)-chicoric acids. *Synth. Commun.* 1998; *28*: 737-740

Zhou, W., Parent, L.J., Willis, J.W., Resh, M.D. Ide,tification of a membrane-binding domain within the amino-terminal region of human immunodeficiency virus type 1 Gag protein which interacts with acidic phospholipids. *J. Virol.* 1994; *68*: 2556-2569

What is claimed is:

1. A method for determining susceptibility of an HIV isolate from HIV infected cell to anti-HIV activity of compounds, comprising:
   - Excising a gp120, gp41, gp160, integrase or gag encoding polynucleotide sequence from a proviral molecular HIV clone;
   - Recirculizing the gp120, gp41, gp160, integrase or gag deleted proviral molecular HIV clone with a base pair linker containing selected restriction sites;
   - DNA extraction of proviral DNA or RNA extraction of HIV virus;
   - PCR amplifying a gp120, a gp41, a gp160, an integrase or a gag encoding sequence from HIV infected cell;
   - Linearising the gp120, gp41, gp160, integrase or gag deleted clone by selected restriction enzymes;
   - Co-transfecting of an eukaryotic cell with the amplified DNA isolates encoding gp120, gp41, gp160, integrase or gag and with said linearised proviral molecular HIV clone (vector) which is deleted in the corresponding encoding polynucleotide sequence and culturing the co-transfected cell whenever homologous recombination is obtained;
   - Harvesting the chimeric HIV stock containing the HIV polynucleotide sequence encoding gp120, gp41, gp160, integrase or gag;
   - Evaluating phenotypic resistance of said chimeric HIV stock to the compounds with said anti-HIV activity or to combinations of the compounds with said anti-HIV activity.

2. A method as claimed in claim 1, wherein in recirculisation of the gp120, gp41, gp160, integrase or gag deleted proviral molecular HIV clone is carried out by a base pair linker containing selected restriction sites and said base pair having a sequence which can homologously recombine with the gp120, gp41, gp160, integrase or gag or mutant or fragment thereof.

3. The method of claim 1, wherein the gp120 deleted proviral molecular HIV clone is recirculised by ligation into the vector of a base pair linker which contains a Sal I and Mam I restriction site which can homologously recombine with the gp120 gene or a mutant or fragment thereof.

4. The method of claim 1, wherein the gp41 deleted proviral molecular HIV clone is recirculised by ligation into the vector of a base pair linker which contains a Xba I restriction site and which can homologously recombine with the gp41 gene or a mutant or fragment thereof.

5. The method of claim 1, wherein the gp160 deleted proviral molecular HIV clone is recirculised by ligation into the vector of a base pair linker which contains a Sal I and Cell II restriction site and which can homologously recombine with the gp160 gene or a mutant or fragment thereof.

6. The method of claim 1, wherein integrase deleted proviral molecular HIV clone is recirculised by ligation into the vector of a base pair linker which contains a Xba I restriction site and which can homologously recombine with the integrase gene or a mutant or fragment thereof.

7. The method of claim 1, wherein or the gag deleted proviral molecular HIV clone is recirculised by ligation into the vector of a base pair linker which contains a Mlu I restriction site and which can homologously recombine with the gag gene or a mutant or fragment thereof.

8. The method of claim 3, wherein the gp120 deleted proviral molecular HIV clone is recirculised by ligation into the vector of a 23/19 base pair linker.

9. The method of claim 4, wherein the gp41 deleted proviral molecular HIV clone is recirculised by ligation into the vector of a 22/25 base pair linker.

10. The method of claim 5, wherein the gp160 deleted proviral molecular HIV clone is recirculised by ligation into the vector of a 29/28 base pair linker.

11. The method of claim 6, wherein integrase deleted proviral molecular HIV clone is recirculised by ligation into the vector of a 21/14 base pair linker.

12. The method of claim 7, wherein or the gag deleted proviral molecular HIV clone is recirculised by ligation into the vector of a 100/100 base pair linker.

13. A method as claimed in claim 2 or 3, wherein the gp120 deleted proviral molecular HIV clone is recirculised by ligation into the vector of a base pair linker having the sequences 3' GTCACCTGTTTAATCTACA 5' and 5' TCGACAGTGGACAAATTAGATGT 3' or a fragment thereof.

14. A method as claimed in claim 2 or 4, wherein the gp41 deleted proviral molecular HIV clone is recirculised by ligation into the vector of a base pair linker having the sequence 3' AGTAGATCTCTTACTCTGCTCGACT 5' and 5' TCATCTAGAGAATGAGACGAGC 3' or a fragment thereof.

15. A method as claimed in claim 2 or 5, wherein the gp160 deleted proviral molecular HIV clone is recirculised by ligation into the vector of a base pair linker having the sequence 3' GACATTCCCTTTCTTACTCTGCTCGACT 5' and 5' TCGACTGTAAGGGAAAGAATGAGACGAGC 3 or a fragment thereof'.

16. A method as claimed in claim 2 or 6, wherein integrase deleted proviral molecular HIV clone is recirculised by ligation into the vector of a base pair linker having the sequence 3' AGATCTCAGAGGTA 5' and 5' CCGGTCTAGAGTCTCCATAGA 3'.

17. A method as claimed in claim 2 or 7, wherein or the gag deleted proviral molecular HIV clone is recirculised by ligation into the vector of a base pair linker having the sequence 5' CGC GCA CGG CAA GAG GCG AGG GGC GGC GAC TGG TGA

GTA CGC CAA AAA TTT TGA CTA GCG GAG GCT AGA AGG AGA GAG ACG CGT GAC ATA GCA GGA ACT A 3'and 3' GT GCC GTT CTC CGC TCC CCG CCG CTG ACC ACT CAT GCG GTT TTT AAA ACT GAT CGC CTC CGA TCT TCC TCT CTC TGC GCA CTG TAT CGT CCTTGA TGA TC 5' or a fragment thereof.

18. The method according to the claim 1 or 2, wherein the deleted proviral molecular HIV clones are produced by excising a gp120 encoding polynucleotide sequence from a proviral molecular HIV clone by digestion of said proviral molecular HIV clone by restriction enzymes Sal I and Mam I and recirculising by ligating a base pair containing Sal I and Mam I restriction sites into said proviral molecular clone.

19. The method according to the claim 1 or 2, wherein the deleted proviral molecular HIV clones are produced by excising a gp41 encoding polynucleotide sequence from said proviral molecular HIV clone by digestion of said proviral molecular HIV clone by restriction enzymes Mam I and Cell II and recirculising by ligating a base pair containing a Xba I restriction site into said proviral molecular clone.

20. The method according to the claims 1 or 2, wherein the deleted proviral molecular HIV clones are produced by excising a gp160 encoding polynucleotide sequence from said proviral molecular HIV clone by digestion of said proviral molecular HIV clone by restriction enzymes Sal I and Cell II and recirculising by ligating a base pair containing the Sal I and Cell II restriction sites into said proviral molecular clone.

21. The method according to the claim 1 to 2, wherein the deleted proviral molecular HIV clones are produced by excising an integrase encoding polynucleotide sequence from said proviral molecular HIV clone by digestion of said proviral molecular HIV clone by restriction enzymes Pin A1 and Van 91 I and recirculising by ligating a base pair containing the Sal I and Cell II restriction sites into said proviral molecular clone.

22. The method according to the claim 1 or 2, wherein the deleted proviral molecular HIV clones are produced by excising a gag encoding polynucleotide sequence from said proviral molecular HIV clone by digestion of said proviral molecular HIV clone by restriction enzymes BssH II and Spe I and recirculising by ligating a base pair containing the Mlu I restriction site into said proviral molecular clone.

23. The method according to the claim 1 or 2, wherein the deleted proviral molecular HIV clones are produced by excising a gag/gp160 encoding polynucleotide sequence from said proviral molecular HIV clone by digestion of said gp160 deleted proviral molecular clone by restriction enzymes BssH II and Spe I and recirculising by ligating a base pair containing the Mlu I restriction site into said proviral molecular clone.

24. The method according any of the claims 1 to 23, wherein the gp120 deleted clone is linearised by Sal I digestion, the gp41 deleted clone is linearised by Xba I digestion, the gp160 deleted clone is linearised by Sal I digestion or the integrase deleted clone is linearised by Xba I digestion, the gag deleted clone by Mlu I digestion or the gag/gp160 deleted clone by Mlu I digestion.

25. The method according to any of the claims 1, 2, 3, 8, 13 or 24, wherein the gp120 encoding sequence from HIV infected cell are PCR amplified using at least one primer selected from the group the of gp120 primers AV310 (5'-AGC AGG ACA TAA T/CAA GGT AGG-3', AV311 (5'GGA GAA GTG AAT TAT ATA AG/AT ATA AAG TAG-3', AV312 (5'-AGA A/GGA C/TAG ATG GAA CAA GCC CCA G-3' or AV313 (5'-GAC CTG GAG GAG GAA/G ATA TGA G/AGG A-3'.

26. The method according to any of the claims 1, 2, 4, 9, 14, 19 or 24 wherein the gp41 encoding sequence from HIV infected cell are PCR amplified using at least one primer selected from the group the of gp41 primers AV320 (5'-ATT GTA/G GAG GA/GG AAT TTT TCT ACT G-3' and AV321 (5' -TTG CTA/G CTT GTG ATT GCT/C CCA TG-3'.

27. The method according to any of the claims 1, 2, 5, 10, 15, 20 or 24, wherein the gp160 encoding sequence from HIV infected cell are PCR amplified using at least one primer selected from the group of gp160 primers AV310 (5'-AGC AGG ACA TAA T/CAA GGT AGG-3' and AV319 (5' -GCT G/C CC TTA/G TAA GTC ATT GGT CT-3'.

28. The method according to any of the claims 1, 2, 6, 11, 16, 21, or 24, wherein the integrase encoding sequence from HIV infected cell are PCR amplified using at least one primer selected from the group of the integrase primers MW3 (5'-TAT GTA GGA/G TCT GAC/T TTA GAA ATA GGG-3' and MW4 (5'-TAA CAC TAG GCA A/GAG GTG GCT T-3'.

29. The method according to any of the claims 1, 2, 7, 12, 17, 22 or 24, wherein the gag-encoding sequence from HIV infected cell are PCR amplified using at least one primer selected from the group of the GAG primers AV14 (5'-CTC TCT CGA CGC AGG ACT CGG CTT GCT GAA-3') and BVR2 (5'-GCC AG/AA TC/TT TCC CTA AAA AAT TAG CC-3').

30. A method as claimed in any of the claims 1 to 29, wherein the proviral molecular HIV clone consists of the plasmid pUC18 with the complete HIV genome and flanked with chromosomal DNA.

31. A method as claimed in any of the claims 1 to 29, wherein the proviral molecular HIV clone is pNL4.3.

32. A method as claimed in any of the claims 1 to 31, wherein the co-transfected cell is an eukaroytic cell.

33. A method as claimed in any of the claims 1 to 31, wherein the co-transfected cell is a human cell.

34. A method as claimed in any of the claims 1 to 31, wherein the co-transfected cell is a MT-4 cell or a peripheral mononuclear cell.

35. The method of any of the claims 1 to 34, further comprising sequencing of the gp120-encoding region, of the gp41-encoding region, of the gp160-encoding region, of the integrase-encoding region or of the gag-encoding region.

36. The method of claims 35, wherein the gp120 gene were sequenced using al least one or several of the group of gp120 sequencing primers consisting of AV304 (5'-ACA TGT GGA AAA ATG ACA TGG T-3' , AV305 (5'-GAG TGG GGT TAA TTT TAC ACA TGG-3', AV306 (5'-TGT CAG CAC AGT ACA ATG TAC ACA-3', AV307 (5'-TCT TCT TCT GCT AGA CTG CCA T-3', AV308 (5'-TCC TCA GGA GGG GAC CCA GAA ATT-3' , AV309 (5'-CAG TAG AAA AAT TCC CCT CCA CA-3' and AV313 (5'-GAC CTG GAG GAG GAA/G ATA TGA G/AGG A-3'.

37. The method of claims 35, wherein the gp41 gene were sequenced using at least one or several primers of the group of the gp41 sequencing primers consisting of AV322 (5'-AAG CAA TGT ATG CCC CTC C-3', AV323 (5'-CTG CTC CC/TA AGA ACC CAA-3', AV324 (5'-GGC AAA GAG AAG AGT GGT-3' AV325 (5'-GTA TCT TTC CAC AGC TAG-3', AV326 (5'-TTG GGG T/CTG CTC TGG AAA AC-3', AV327 (5'-TTT TAT ATA CCA CAG CCA-3', AV328 (5'-ATA ATG ATA GTA GGA GG-3', AV329 (5'-GTC CCA GAA GTT CCA CA-3', AV330 (5'-GGA G/ACC TGT GCC TCT TCA-3' and AV331 (5'-TCT CAT TCT TTC CCT TA-3'.

38. The method of claims 35, wherein the wherein the gp160 gene were sequenced using al least one or several of the group of the gp120 sequencing primers and using al least one or several of the group of gp41 sequencing primers.

39. The method of claims 35, wherein the p17 and p24 encoding regions of the gag gene were sequenced using al least one or several of the primers AV13 (5'-CTG CGA ATC GTT CTA GCT CCC TGC TTG CCC -3'), AV26 (5'-GCT ATG TCA CTT

CCC CTT GGT TCT C -3'), AV54 (5'-GAG ACC ATC AAT GAG GAA GCT GC-3'), AV58, (5'-GGC TAA TTT TTT AGG-3'), AV159 (5'-GGG GTT AAA TAA AAT AGT AAG-3'), AV103 (5'-GCC ATA TCA CCT AGA ACT TT-3'), GAG-1 (5'-AGA AAC ATC AGA AGG CTG TAG-3'), BVR3 (5'-TTT CCA ACA GCC CTT TTT CCT AG-3'), BVR4 (5'-AGA CAC CAA G/AGA AGC-3'), BVR5 (5'-TCC T/CTC TGA TAA TGC TG-3') and BVR6 (5'-TAG AAG AAA TGA TGA CAG C-3').

40. The method of claims 35, wherein the integrase gene were sequenced using at least one or several of the primers: IN-PCRA (5'-GGA GGA AAT GAA CAA GTA GAT-3', HP4392C (5'-TCT ACT TGT CCA TGC ATG GCTTC-3', IN-SEQ1 (5'-TTA AGA TGT TCA GCC TGA TCT-3' , IN-SEQ2 (5'- CAT TGT CTG TAT GTA CTG TTT-3' , IN-SEQ3 (5'- GGA TAT ATA GAA GCA GAA GTA A-3' , IN-SEQ4 (5'-GAA CAT CTT AAG ACA GCA GTA-3' , IN-SEQ5 (5'- AAG CTC CTC TGG AAA GGT GAA-3' and IN-PCRB (5'- CCT TGA AAT ATA CAT ATG GTG-3').

41. The method of claim 1 to 40, further comprising comparing data of the phenotypic resistance of said HIV stock containing the selected HIV polynucleotide sequence of said HIV isolate to anti-HIV activity of said compounds or of said combinations of said compounds with data of the phenotypic resistance of said HIV stock containing a wild type HIV polynucleotide sequence to anti-HIV activity of said compounds or of said combinations of said compounds.

42. The method of claim 41, further comprising repeating said resistance test for al least one more time and constructing at least two more data sets.

43. The method of claim 42, further comprising calculation of the fold decrease in susceptibility to said compound with anti-HIV activity or to combinations of said compounds of the chimeric viruses in comparison to the wild-type chimeric strains.

44. The method of any of the claim 1 to 43, wherein said DNA extraction of proviral DNA or RNA extraction of HIV virus is obtainable from an HIV patient.

45. The method of any of the claims 1 to 45, wherein said HIV isolate is selected from an in vitro culture of cells infected with a population of a HIV virus strain that was cultured in the presence of compounds or combinations of compounds with putative or with established anti-HIV activity.

46. The use of the method of claim 45 to study molecular target and resistance profile of action of said compounds with anti-HIV activity.

47. The use of method of claim 44, to adapt the chemotherapy administered to an HIV patient.

48. A genetic information data set on anti-HIV resistance, obtainable by the method claim 44 or 46.

49. The genetic information data set of claim 48, stored in the memory of computer controlling a gene-sequencing machine to screen for mutations in genes of HIV isolates.

50. The use of said genetic information data set of claim 47 or 49 that confer resistance to said anti-HIV compound to influence anti-HIV therapy.

51. The method according to claim 1 to claim 45, wherein the phenotypic sensitivity of said recombined viruses to inhibitors inducing mutations in HIV envelope proteins is assessed.

52. A method according to claim 1 to claim 45, wherein the phenotypic sensitivity of recombined viruses containing two resistant virus segments, i.e. (gp160 and p17/p24) to inhibitors inducing mutations in HIV envelope proteins (gp120, gp41, gp160) is assessed.

53. A method according to claim 1 or claim 45, wherein the phenotypic sensitivity of said recombined viruses to inhibitors of HIV integrase (IN, p32) is assessed.

54. A kit for determining susceptibility of an HIV isolate from HIV infected cell to anti-HIV activity of compounds, wherein a amplification primer cocktail comprises primers of the sequence: AV310 (5'-AGC AGG ACA TAA T/CAA GGT AGG-3' , AV311 (5'GGA GAA GTG AAT TAT ATA AG/AT ATA AAG TAG-3', AV312 (5'-AGA A/GGA C/TAG ATG GAA CAA GCC CCA G-3', AV313 (5'-GAC CTG GAG GAG GAA/G ATA TGA G/AGG A-3', AV320 (5'-ATT GTA/G GAG GA/GG AAT TTT TCT ACT G-3', AV321 (5' -TTG CTA/G CTT GTG ATT GCT/C CCA TG-3', AV310 (5'-AGC AGG ACA TAA T/CAA GGT AGG-3', AV319 (5' -GCT G/C CC TTA/G TAA GTC ATT GGT CT-3', MW3 (5'-TAT GTA GGA/G TCT GAC/T TTA GAA ATA GGG-3', MW4 (5'-TAA CAC TAG GCA A/GAG GTG GCT T-3', AV14 (5'-CTC TCT CGA CGC AGG ACT CGG CTT GCT GAA-3') and BVR2 (5'-GCC AG/AA TC/TT TCC CTA AAA AAT TAG CC-3').

55. The kit of claim 54, further comprising linkers of the group consisting of a base pair linker having the sequences 3' GTCACCTGTTTAATCTACA 5' and 5' TCGACAGTGGACAAATTAGATGT 3' or a fragment thereof, a base pair linker having the sequence 3' AGTAGATCTCTTACTCTGCTCGACT 5' and 5' TCATCTAGAGAATGAGACGAGC 3' or a fragment thereof, a base pair linker having the sequence 3' GACATTCCCTTTCTTACTCTGCTCGACT 5' and 5' TCGACTGTAAGGGAAAGAATGAGACGAGC 3 or a fragment thereof', a base pair linker having the sequence 3' AGATCTCAGAGGTA 5' and 5' CCGGTCTAGAGTCTCCATAGA 3' or a fragment thereof and a base pair linker having the sequence 5' CGC GCA CGG CAA GAG GCG AGG GGC GGC GAC TGG TGA GTA CGC CAA AAA TTT TGA CTA GCG GAG GCT AGA AGG AGA GAG ACG CGT GAC ATA GCA GGA ACT A 3'and 3' GT GCC GTT CTC CGC TCC CCG CCG CTG ACC ACT CAT GCG GTT TTT AAA ACT GAT CGC CTC CGA TCT TCC TCT CTC TGC GCA CTG TAT CGT CCTTGA TGA TC 5' or a fragment thereof.

56. The kit of claim 55, further comprising a MT-4 or peripheral mononuclear cells line.

57. The kit of claim 56, further comprising sequencing primers selected from the group consisting of AV304 (5'-ACA TGT GGA AAA ATG ACA TGG T-3' , AV305 (5'-GAG TGG GGT TAA TTT TAC ACA TGG-3', AV306 (5'-TGT CAG CAC AGT ACA ATG TAC ACA-3', AV307 (5'-TCT TCT TCT GCT AGA CTG CCA T-3', AV308 (5'-TCC TCA GGA GGG GAC CCA GAA ATT-3' , AV309 (5'-CAG TAG AAA AAT TCC CCT CCA CA-3', AV313 (5'-GAC CTG GAG GAG GAA/G ATA TGA G/AGG A-3', AV322 (5'-AAG CAA TGT ATG CCC CTC C-3', AV323 (5'-CTG CTC CC/TA AGA ACC CAA-3', AV324 (5'-GGC AAA GAG AAG AGT GGT-3' AV325 (5'-GTA TCT TTC CAC AGC TAG-3', AV326 (5'-TTG GGG T/CTG CTC TGG AAA AC-3', AV327 (5'-TTT TAT ATA CCA CAG CCA-3', AV328 (5'-ATA ATG ATA GTA GGA GG-3', AV329 (5'-GTC CCA GAA GTT CCA CA-3', AV330 (5'-GGA G/ACC TGT GCC TCT TCA-3', AV331 (5'-TCT CAT TCT TTC CCT TA-3', AV13 (5'-CTG CGA ATC GTT CTA GCT CCC TGC TTG CCC -3'), AV26 (5'-GCT ATG TCA CTT CCC CTT GGT TCT C -3'), AV54 (5'-GAG ACC ATC AAT GAG GAA GCT GC-3'), AV58, (5'-GGC TAA TTT TTT AGG-3'), AV159 (5'-GGG GTT AAA TAA AAT AGT AAG-3'), AV103 (5'-GCC ATA TCA CCT AGA ACT TT-3'), GAG-1 (5'-AGA AAC ATC AGA AGG CTG TAG-3'), BVR3 (5'-TTT CCA ACA GCC CTT TTT CCT AG-3'), BVR4 (5'-AGA CAC CAA G/AGA AGC-3'), BVR5 (5'-TCC T/CTC TGA TAA TGC TG-3'), BVR6 (5'-TAG AAG AAA TGA TGA CAG C-3'), IN-PCRA (5'-GGA GGA AAT GAA CAA GTA GAT-3', HP4392C (5'-TCT ACT TGT CCA TGC ATG GCTTC-3', IN-SEQ1 (5'-TTA AGA TGT TCA GCC TGA TCT-3' , IN-SEQ2 (5'- CAT TGT CTG TAT GTA CTG TTT-3' , IN-SEQ3 (5'- GGA TAT ATA GAA GCA GAA GTA A-3' , IN-SEQ4 (5'- GAA CAT CTT AAG ACA GCA GTA-3' , IN-SEQ5 (5'-AAG CTC CTC TGG AAA GGT GAA-3' and IN-PCRB (5'- CCT TGA AAT ATA CAT ATG GTG-3').

Fig. 1a Construction of the *gp120*-deleted clone

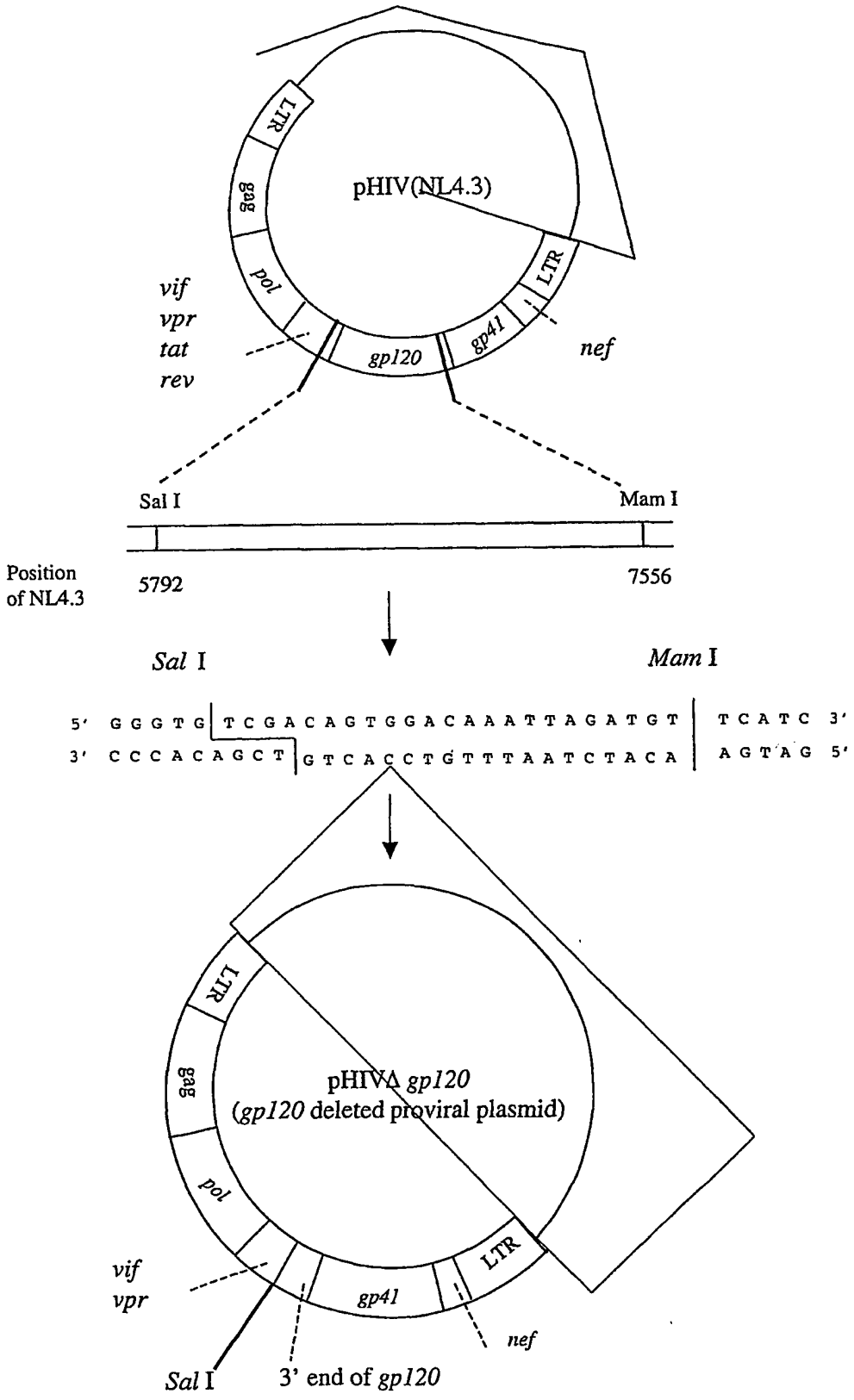

Fig. 1b *gp120*-recombination

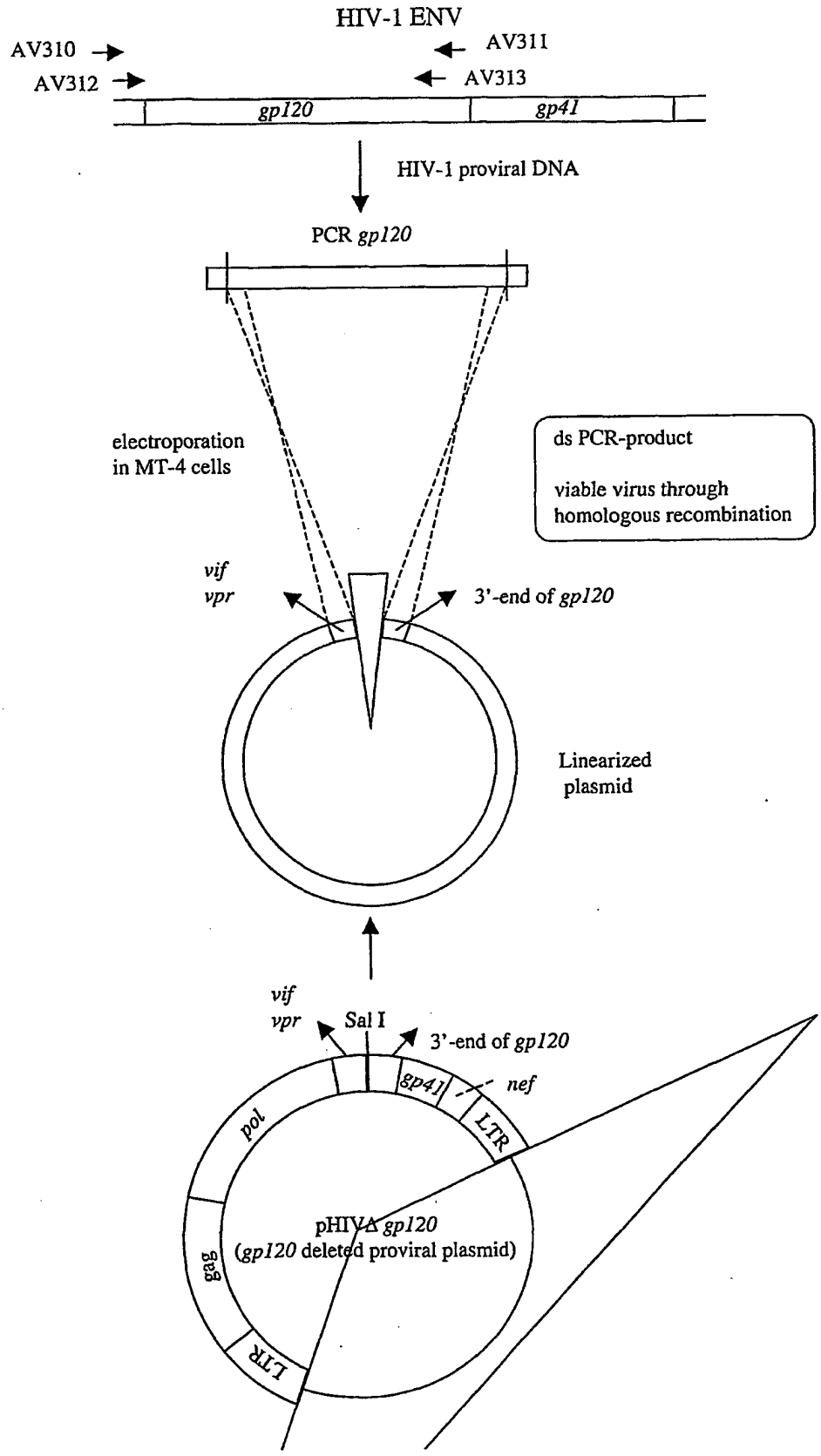

Fig. 2a Construction of the *gp41*-deleted clone

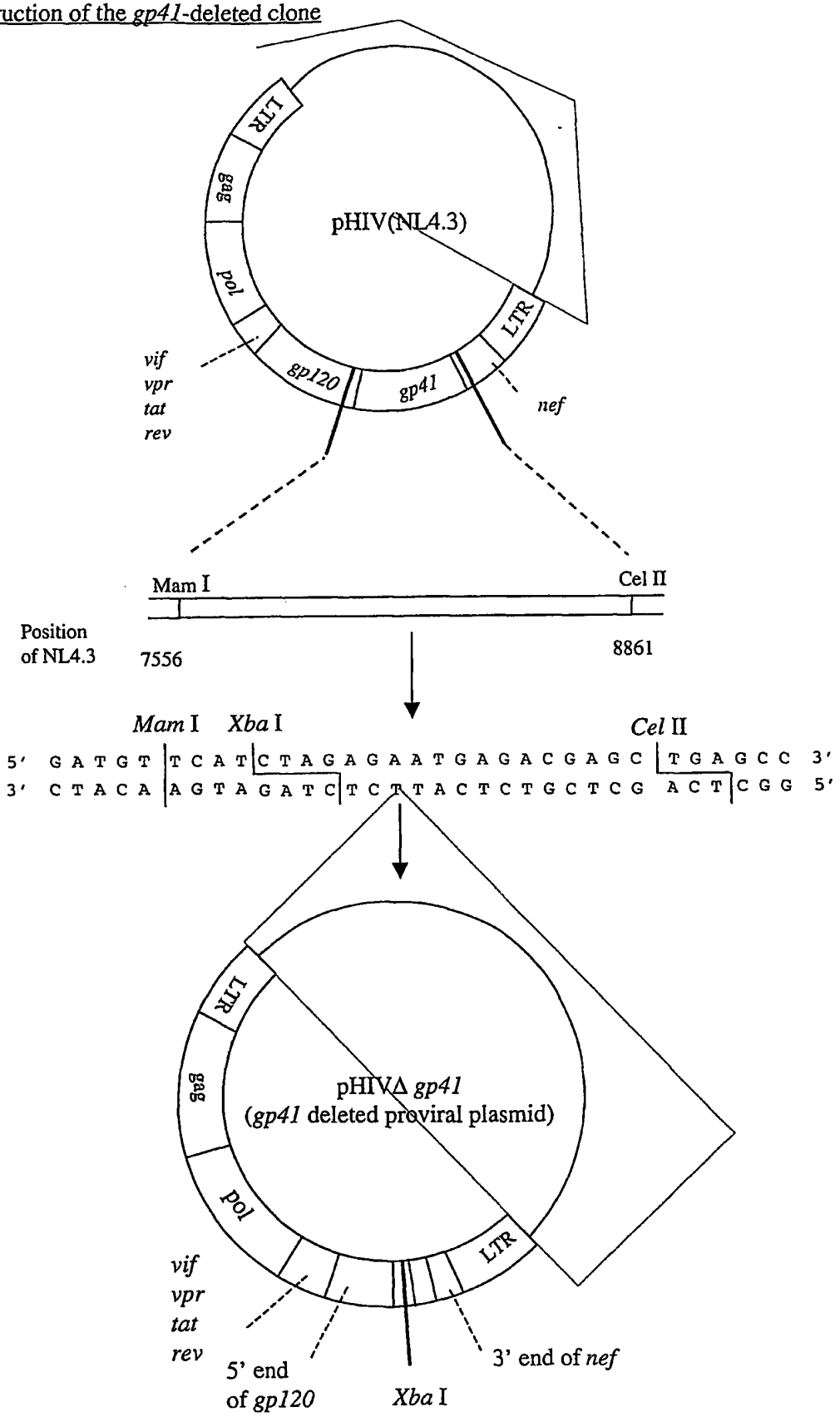

Fig. 2b *gp41*-recombination

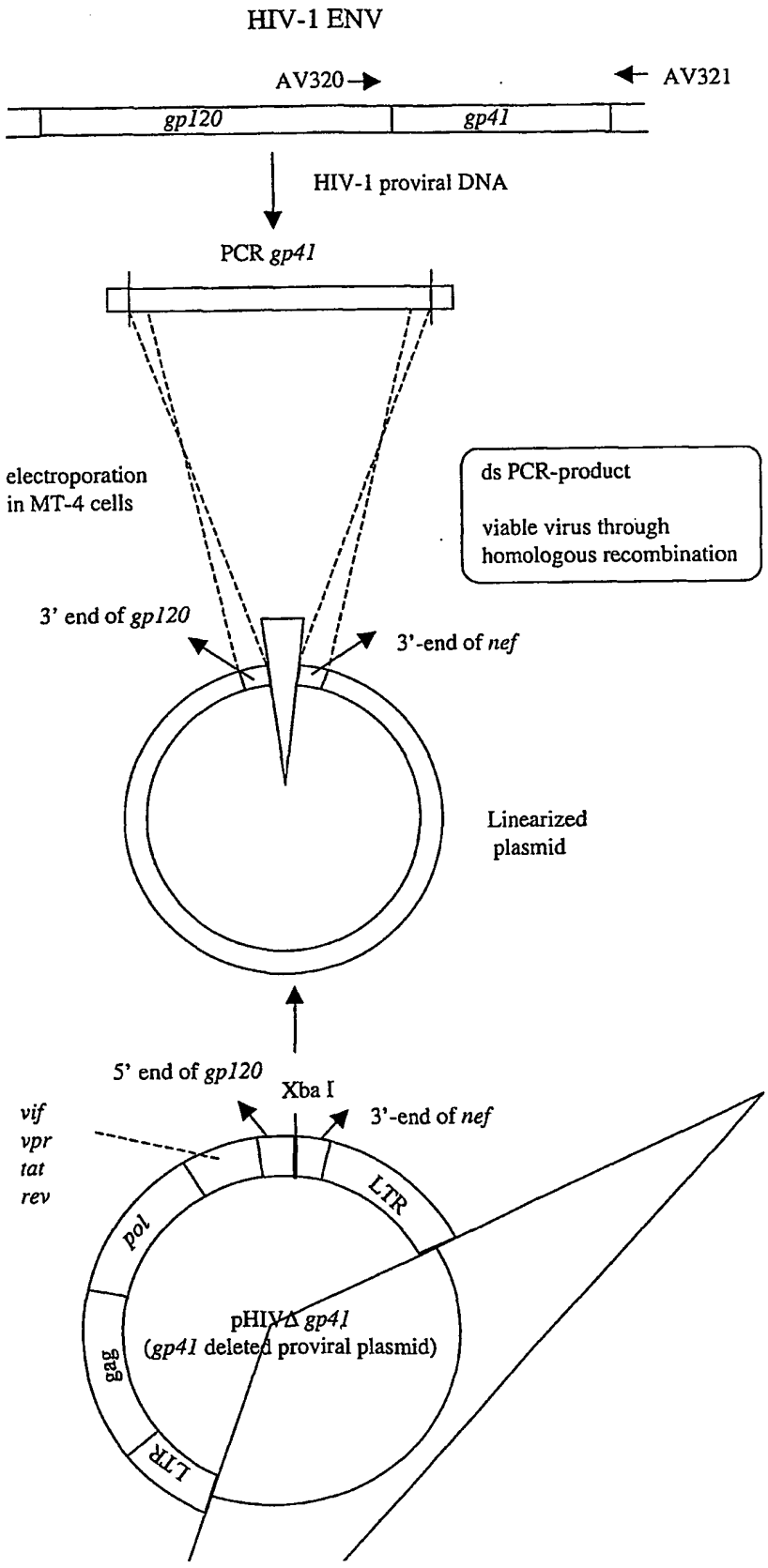

Fig. 3a Construction of the *gp160*-deleted clone

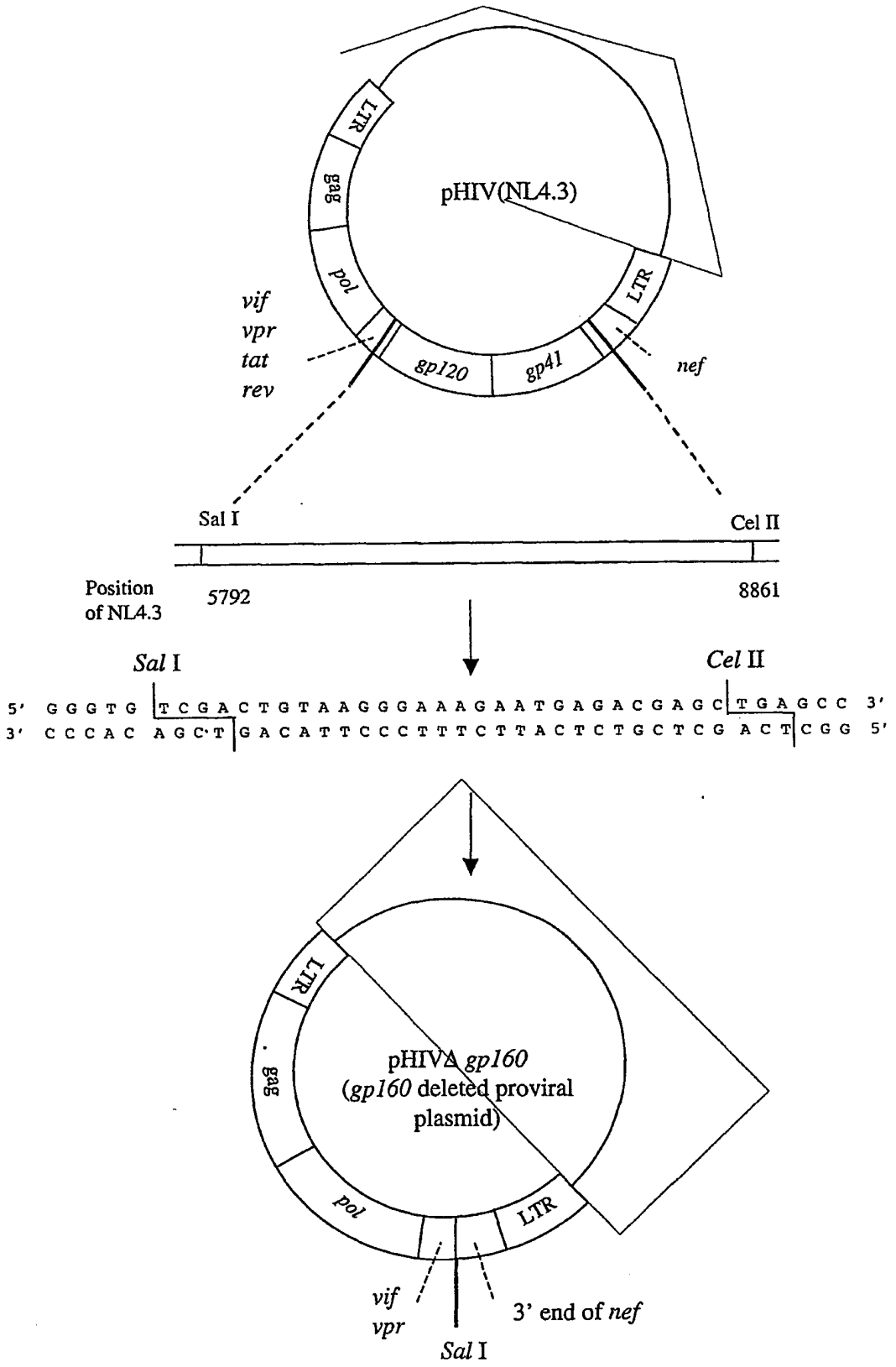

Fig. 3b *gp160*-recombination

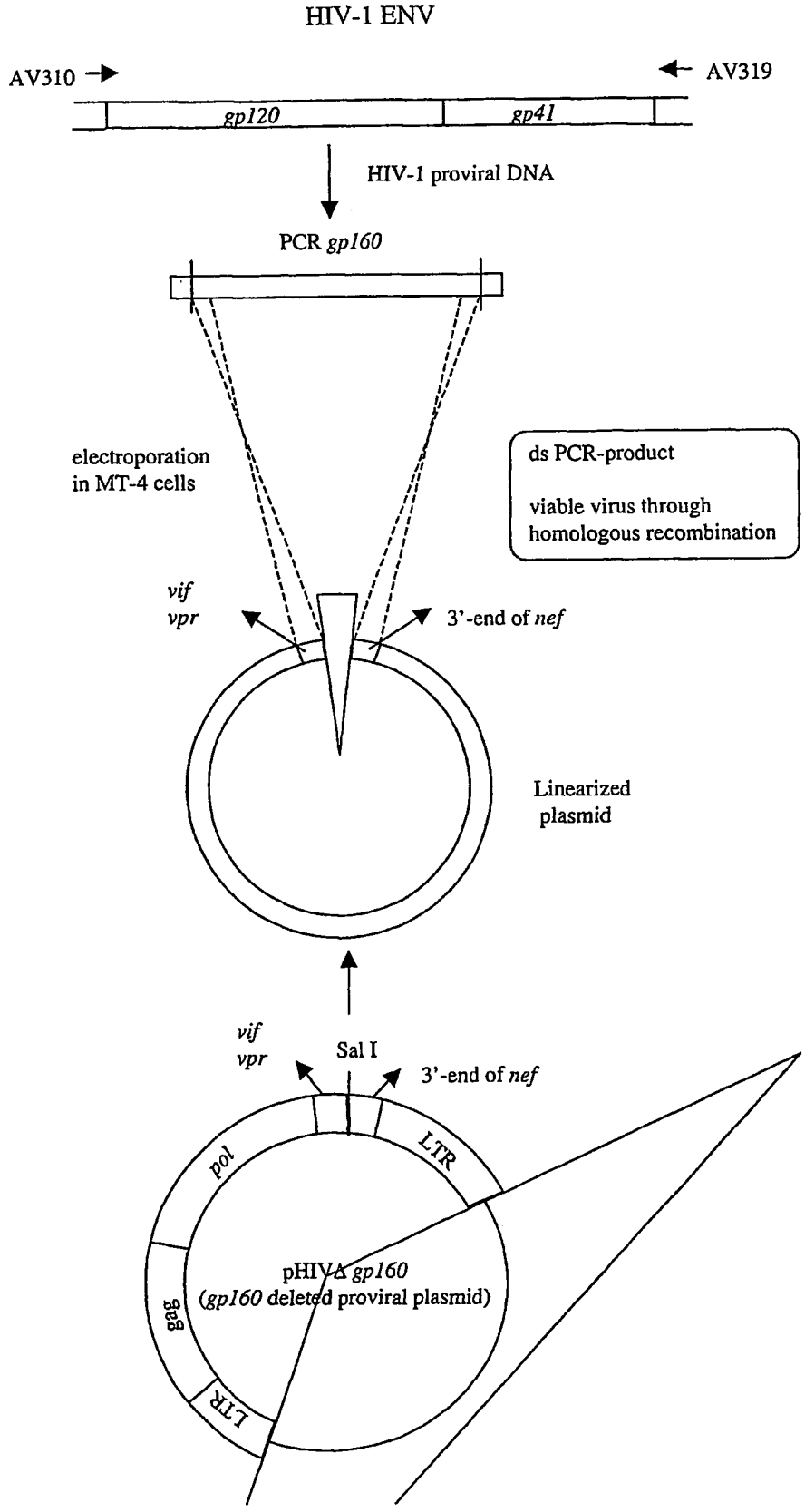

Fig. 4a Construction of the *integrase*-deleted clone

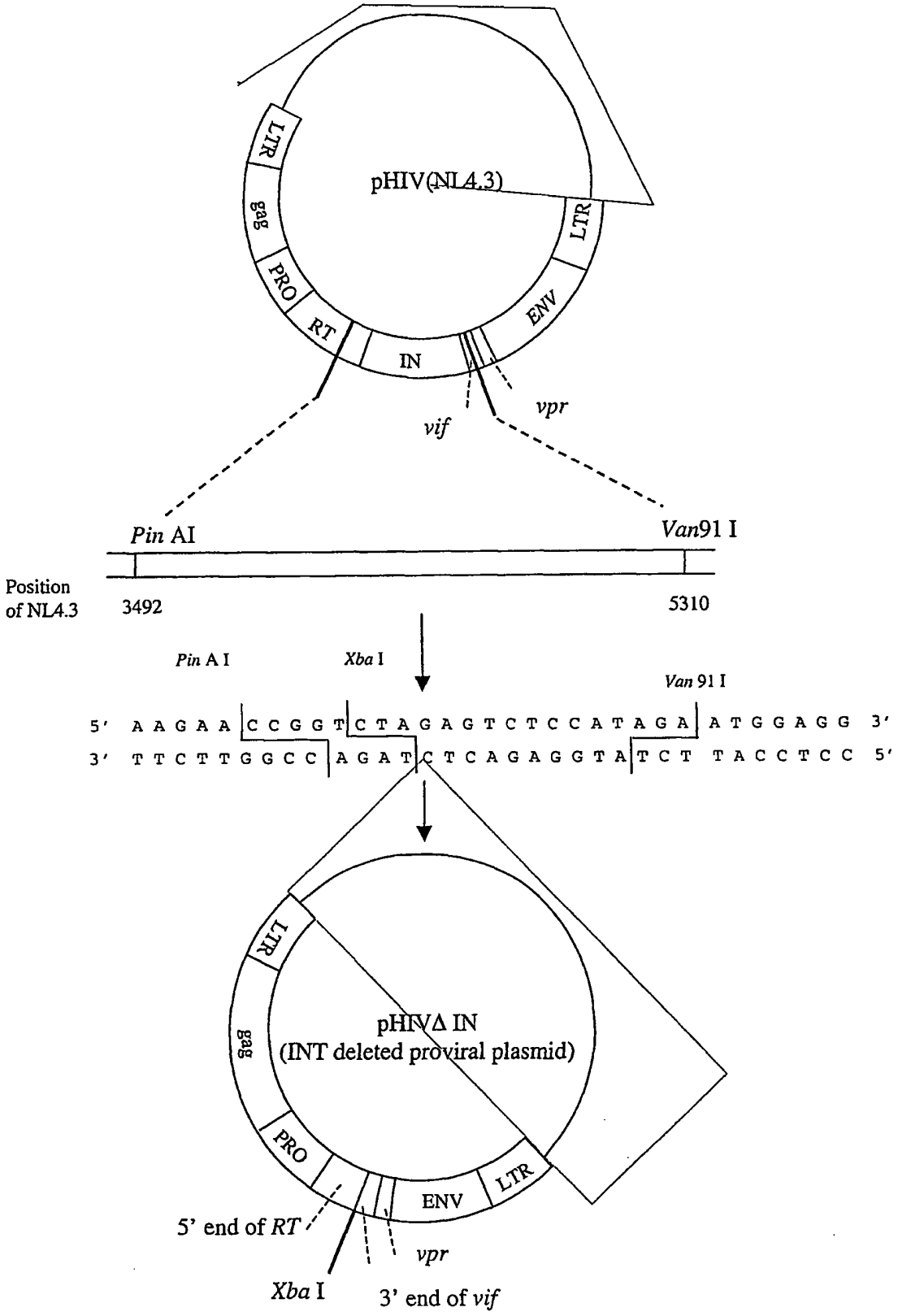

Fig. 4b *Integrase*-recombination

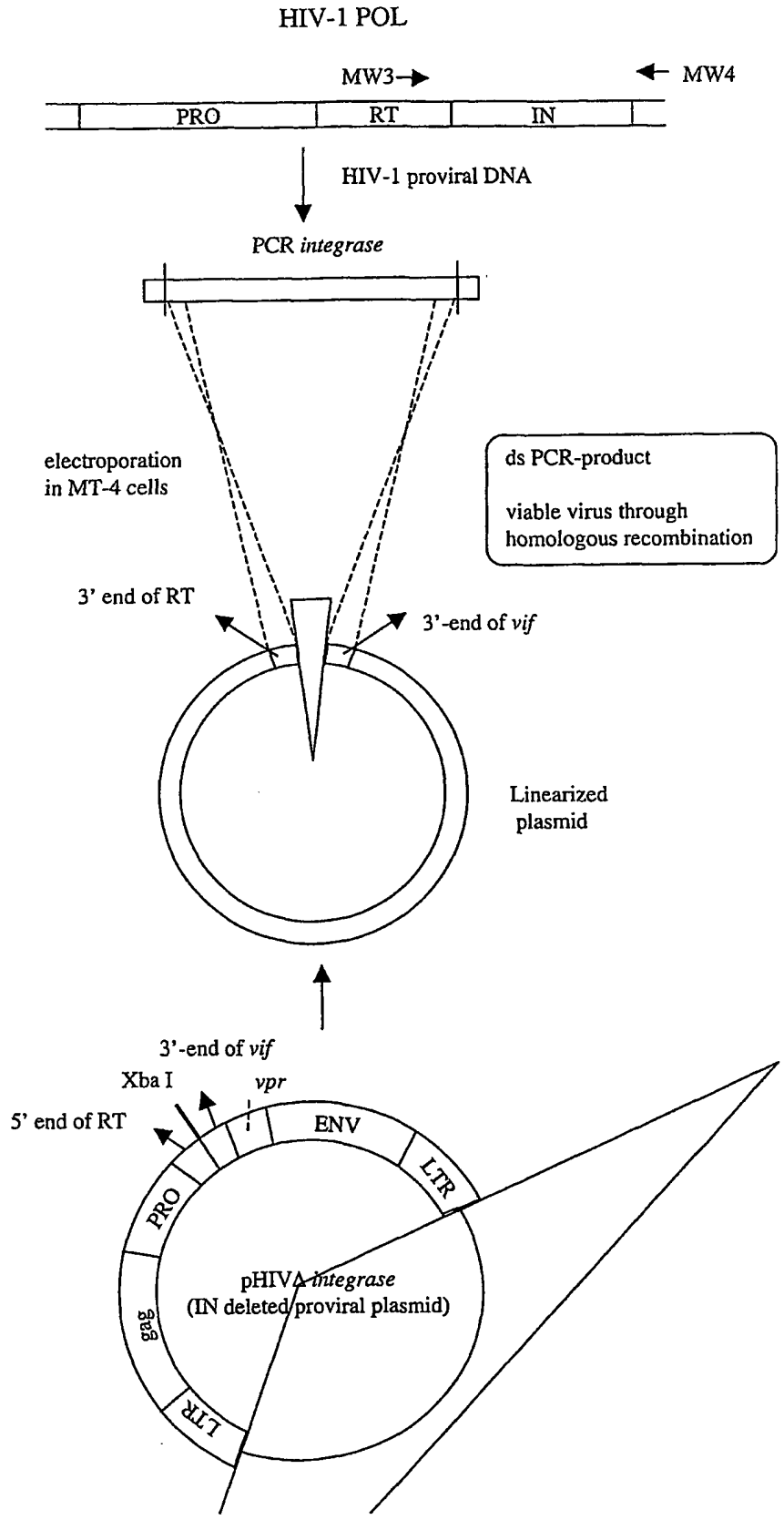

Fig. 5a Construction of the *p17/gp160*-deleted clone

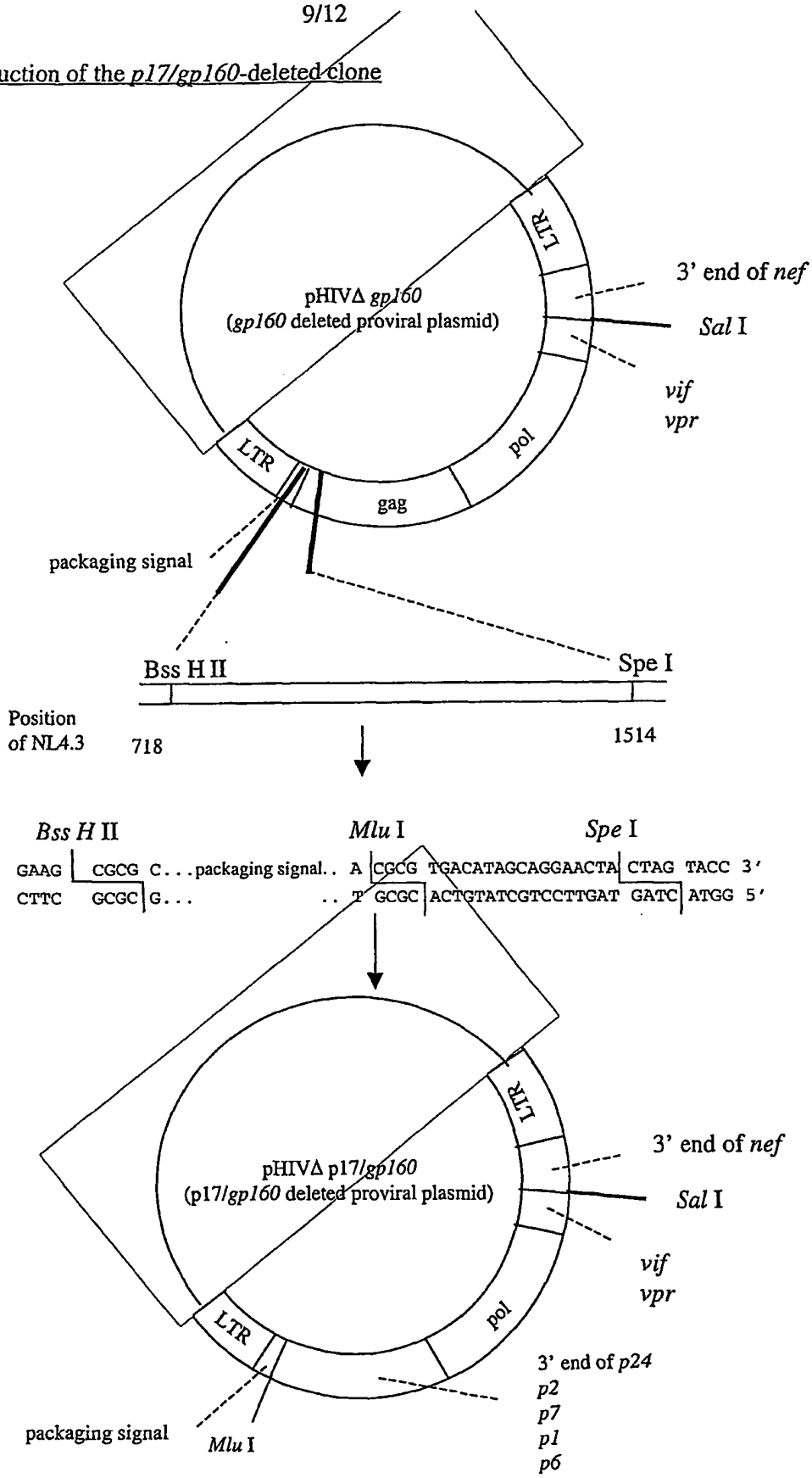

Fig. 5b *p17/gp160*-recombination

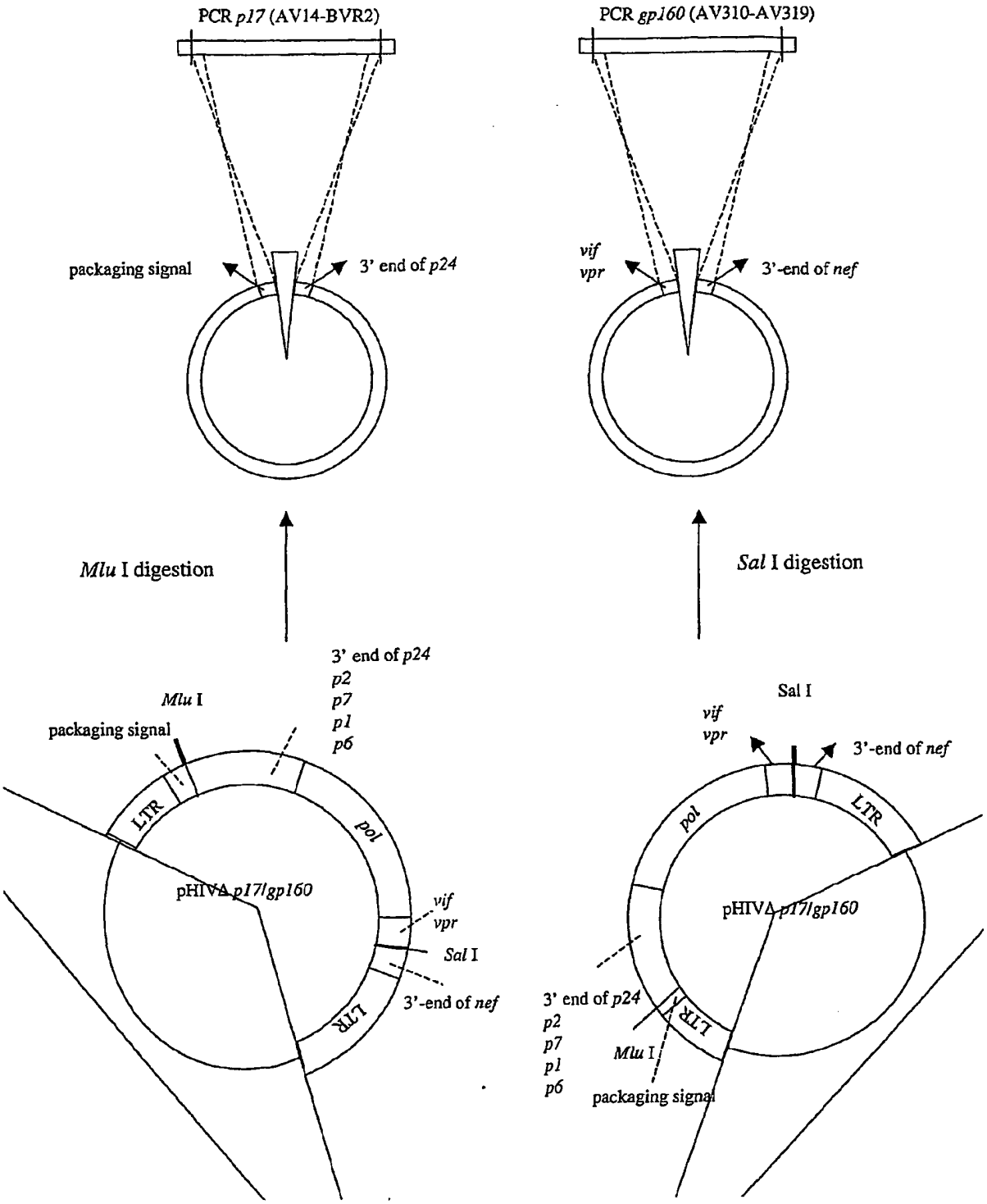

Figure 6. Principle of the PCR amplification of the linker sequence

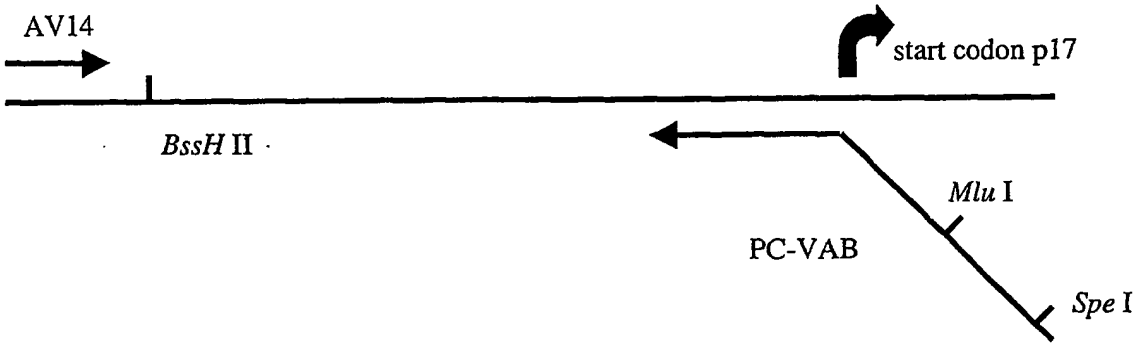

Figure 7. Complete sequence of the linker

```
5'CGC GCA CGG CAA GAG GCG AGG GGC GGC GAC TGG TGA GTA CGC CAA AAA
3'     GT GCC GTT CTC CGC TCC CCG CCG CTG ACC ACT CAT GCG GTT TTT

TTT TGA CTA GCG GAG GCT AGA AGG AGA GAG ACG CGT GAC ATA GCA GGA
AAA ACT GAT CGC CTC CGA TCT TCC TCT CTC TGC GCA CTG TAT CGT CCT
```

Mlu I

```
ACT A          3'
TGA TGA TC     5'
```

non-complementary sequence

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
9 August 2001 (09.08.2001)

PCT

(10) International Publication Number
WO 01/57245 A3

(51) International Patent Classification[7]: C12Q 1/70

(21) International Application Number: PCT/BE01/00017

(22) International Filing Date: 5 February 2001 (05.02.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

0002533.8	4 February 2000 (04.02.2000)	GB
0101011.5	15 January 2001 (15.01.2001)	GB

(71) Applicant *(for all designated States except US)*: **K.U.LEUVEN RESEARCH & DEVELOPMENT** [BE/BE]; Groot Begijnhof, Benedenstraat 59, B-3000 Leuven (BE).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **WITVROUW, Myriam** [BE/BE]; Wandeleerstraat 27, B-3201 Langdorp (BE). **FIKKERT, Valery** [BE/BE]; Nindsebaan 43, B-3140 Keerbergen (BE). **PANNECOUQUE, Christophe** [BE/BE]; Sint-Jansbergsesteenweg 70, B-3001 Leuven (Heverlee) (BE). **CHEREPANOV, Peter** [RU/BE]; Brusselsestraat 128, B-3000 Leuven (BE). **VAN LAETHEM, Kristel** [BE/BE]; Hoogstraat 176, B-2830 Tisselt (BE). **DE CLERCQ, Erik** [BE/BE]; Parklaan 9, B-3360 Lovenjoel (BE). **VANDAMME, Anne-Mieke** [BE/BE]; Beatrijslaan 9, B-3110 Rotselaar (BE). **DEBYSER, Zeger** [BE/BE]; Korbeek-Losestraat 108, B-3001 Heverlee (BE).

(74) Agent: **ROELANTS, Ivo**; K.U.Leuven Research and Development, Groot Begijnhof, Benedenstraat 59, B-3000 Leuven (BE).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

(88) Date of publication of the international search report:
27 June 2002

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/57245 A3

(54) Title: HIV-1 RESISTANCE ASSAY

(57) Abstract: Present invention involves a recombination assay for the HIV envelope genes, *gp120*, *gp41*, and *gp160*. It further involves *env*-deleted proviral clones, the optimization of the PCR amplification of the corresponding *env*-genes and the subsequent sequencing of these genes. These techniques have been applied on several HIV-1(NL4.3) strains selected *in vitro* in the presence of increasing concentrations of inhibitors of HIV entry and evaluated for the phenotypic resistance of these recombined viruses. This phenotypic resistance has been correlated with genotypic resistance. Present invention also involves a recombination assay for the integrase gene.

INTERNATIONAL SEARCH REPORT

Inter tional Application No
PCT/BE 01/00017

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
SEQUENCE SEARCH, EPO-Internal, WPI Data, MEDLINE, BIOSIS, CHEM ABS Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 27480 A (HERTOGS KURT ;PAUWELS RUDI (BE); VIRCO NV (BE); BETHUNE MARIE P DE) 31 July 1997 (1997-07-31) page 4, line 23 -page 5, line 13 page 14, line 12 -page 16, line 5; claims ---	1
Y	MARTINEZ-PICADO J ET AL: "HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 CLONING VECTORS FOR ANTIRETROVIRAL RESISTANCE TESTING" JOURNAL OF CLINICAL MICROBIOLOGY, WASHINGTON, DC; US, vol. 37, no. 9, September 1999 (1999-09), pages 2943-2951, XP002933597 ISSN: 0095-1137 abstract, "Materials and Methods", p. 2945, col. 2, last par.-p. 2947, col. 1, l. 2, table 2 --- -/--	1-57

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 December 2001	22/01/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Luzzatto, E

4

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	DATABASE MEDLINE 'Online! US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US; ESTE J A ET AL: "Human immunodeficiency virus glycoprotein gp120 as the primary target for the antiviral action of AR177 (Zintevir)." retrieved from STN Database accession no. 1998130706 XP002186265 abstract & MOLECULAR PHARMACOLOGY, (1998 FEB) 53 (2) 340-5. ,	1-57

A	HERTOGS ET AL: "A Rapid Method for Simultaneous Detection of Phenotypic Resistance to Inhibitors of Protease and Reverse Transcriptase in Recombinant Human Immunodeficiency Virus Type 1 Isolates from Patients Treated with Antiretroviral Drugs" ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, AMERICAN SOCIETY FOR MICROBIOLOGY, WASHINGTON, DC, US, vol. 42, no. 2, February 1998 (1998-02), pages 269-276, XP002137814 ISSN: 0066-4804 the whole document	1

A	ADACHI A ET AL: "PRODUCTION OF ACQUIRED IMMUNODEFICIENCY SYNDROME-ASSOCIATED RETROVIRUS IN HUMAN AND NONHUMAN CELLS TRANSFECTED WITH AN INFECTIOUS MOLECULAR CLONE" JOURNAL OF VIROLOGY, NEW YORK, US, US, vol. 59, no. 2, August 1986 (1986-08), pages 284-291, XP000870136 ISSN: 0022-538X cited in the application the whole document	1

	-/--	

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 25 November 1999 (1999-11-25) HUNG CHIA-SUEI ET AL: "Relationship between productive HIV-1 infection of macrophages and CCR5 utilization." Database accession no. PREV200000104601 XP002186266 abstract & VIROLOGY, vol. 264, no. 2, 25 November 1999 (1999-11-25), pages 278-288, ISSN: 0042-6822	1-57
A	US 5 686 272 A (MARSHALL RONALD L ET AL) 11 November 1997 (1997-11-11) SEQ ID 17 example 6	54
A	WO 93 11230 A (DYNAL AS) 10 June 1993 (1993-06-10) abstract, claim 11(seq. 13)	54
A	CHO MICHAEL W ET AL: "Identification of determinants on a dualtropic human immunodeficiency virus type 1 envelope glycoprotein that confer usage of CXCR4." JOURNAL OF VIROLOGY, vol. 72, no. 3, March 1998 (1998-03), pages 2509-2515, XP002186429 ISSN: 0022-538X the whole document	1
A	US 5 427 930 A (BIRKENMEYER LARRY G ET AL) 27 June 1995 (1995-06-27) SEQ ID 9 claims	54
A	WO 98 51808 A (BERKHOUT BENJAMIN ;AMC AMSTERDAM (NL); DAS ATZE TAEDE (NL)) 19 November 1998 (1998-11-19) page 7, line 24 -page 8, line 34 page 23, line 20 - line 30; claims	1,54
A	US 5 599 662 A (RESPESS RICHARD A) 4 February 1997 (1997-02-04) claims, SEQ ID 2	4
A	EP 0 591 914 A (BEHRINGWERKE AG) 13 April 1994 (1994-04-13) claims,SEQ ID 24	54

-/--

4

INTERNATIONAL SEARCH REPORT

Inter ional Application No
PCT/BE 01/00017

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,A	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; September 2000 (2000-09) PLUYMERS WIM ET AL: "Viral entry as the primary target for the anti-HIV activity of chicoric acid and its tetra-acetyl esters." Database accession no. PREV200000461434 XP002186430 abstract & MOLECULAR PHARMACOLOGY, vol. 58, no. 3, September 2000 (2000-09), pages 641-648, ISSN: 0026-895X ---	1
A	PANNECOUQUE C. ET AL.: "Viral entry as the primary target of anti-HIV activity of chicoric acid and its ttra-acetyl esters" ANTIVIRAL RESEARCH, vol. 46, no. 1, April 2000 (2000-04), page A36 XP001038484 abstract -----	1

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claim 54 relates to a kit comprising "primers of the sequence:...". Claim 55 is dependent on claim 54 and relates to 10 linkers and fragments (of undefined length) thereof, whereas claim 57 (which depends on claims 54-56) relates to a large number of additional primers. In view of the very large number of possible combinations encompassed by claims 55 and 57, and of the fact that no meaningful search can be performed for fragments of undefined length, the search with respect of the claimed kit has been carried out only insofar as related to the sequences of claim 54.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/BE 01/00017

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9727480	A	31-07-1997	AU	717755 B2	30-03-2000
			AU	1316897 A	20-08-1997
			BG	102710 A	31-03-1999
			BR	9707204 A	28-12-1999
			CN	1209875 A	03-03-1999
			CZ	9802335 A3	16-06-1999
			EP	0877937 A1	18-11-1998
			WO	9727480 A1	31-07-1997
			NO	983300 A	25-09-1998
			NZ	325912 A	28-01-1999
			PL	328069 A1	04-01-1999
			SK	100298 A3	11-02-1999
			TR	9801443 T2	21-10-1998
			US	6221578 B1	24-04-2001
US 5686272	A	11-11-1997	AU	4387193 A	30-12-1993
			CA	2136764 A1	09-12-1993
			EP	0642592 A1	15-03-1995
			JP	8503600 T	23-04-1996
			WO	9324656 A1	09-12-1993
WO 9311230	A	10-06-1993	AU	3084492 A	28-06-1993
			WO	9311230 A1	10-06-1993
US 5427930	A	27-06-1995	CA	2111962 A1	07-01-1993
			DE	69229999 D1	21-10-1999
			DE	69229999 T2	04-05-2000
			EP	0596918 A1	18-05-1994
			ES	2138974 T3	01-02-2000
			JP	6508750 T	06-10-1994
			US	5516663 A	14-05-1996
			WO	9300447 A1	07-01-1993
			US	5453355 A	26-09-1995
			AT	137269 T	15-05-1996
			AU	635105 B2	11-03-1993
			AU	7000791 A	01-08-1991
			CA	2035010 A1	27-07-1991
			DE	69118930 D1	30-05-1996
			DE	69118930 T2	09-01-1997
			EP	0439182 A2	31-07-1991
			ES	2089038 T3	01-10-1996
			JP	1980321 C	17-10-1995
			JP	4211399 A	03-08-1992
			JP	6036760 B	18-05-1994
			KR	9513953 B1	18-11-1995
			KR	9602235 B1	13-02-1996
			US	5573907 A	12-11-1996
			US	5792607 A	11-08-1998
WO 9851808	A	19-11-1998	EP	0878547 A1	18-11-1998
			AU	7553798 A	08-12-1998
			EP	0972064 A1	19-01-2000
			WO	9851808 A1	19-11-1998
US 5599662	A	04-02-1997	CA	2169315 A1	18-08-1996
			EP	0727497 A1	21-08-1996
			JP	8242898 A	24-09-1996

INTERNATIONAL SEARCH REPORT

..formation on patent family members

Inter ·ional Application No
PC·/BE 01/00017

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0591914 A	13-04-1994	DE 4233646 A1	07-04-1994
		DE 4235718 A1	28-04-1994
		DE 4244541 A1	07-07-1994
		DE 4318186 A1	05-01-1995
		AT 174382 T	15-12-1998
		AU 677225 B2	17-04-1997
		AU 4880093 A	21-04-1994
		CA 2107732 A1	07-04-1994
		DE 59309207 D1	21-01-1999
		DK 591914 T3	16-08-1999
		EP 0591914 A2	13-04-1994
		EP 0890642 A2	13-01-1999
		ES 2126620 T3	01-04-1999
		JP 6225760 A	16-08-1994
		JP 2000312592 A	14-11-2000
		US 6277561 B1	21-08-2001
		US 5840480 A	24-11-1998
		US 5759770 A	02-06-1998
		US 5770427 A	23-06-1998
		ZA 9307371 A	29-04-1994